AD_____

Award Number:
W81XWH-06-1-0423

TITLE:
The Role of Akt and its Substrates in Resistance of Breast Cancer to Trastuzumab

PRINCIPAL INVESTIGATOR:
Christian Daniel Young, B.A.

CONTRACTING ORGANIZATION:
University of Colorado Health Sciences Center
Aurora, CO 80045-0508

REPORT DATE:
March 2009

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From - To)*
31-03-2009	Final Summary	1 March 2006 – 28 Feb 2009

4. TITLE AND SUBTITLE
The Role of Akt and its Substrates in Resistance of Breast Cancer to Trastuzumab

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-06-1-0423

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Christian Daniel Young

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Colorado
Aurora, CO 80045-0508

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The broad scope aim of research conducted under this grant was to determine the role of Akt in ErbB2 tumorigenesis. The first two tasks aimed to determine substrates of Akt that are targets of trastuzumab and the results of these studies demonstrated that trastuzumab does not strongly induce apoptosis, and as such, targets downstream of Akt are not affected by trastuzumab. In the third task, we determined that overexpression of constitutively active Akt in the tumors of MMTV-c-ErbB2 mice accelerates mammary tumorigenesis. This acceleration is observed in the context of a reduced requirement for plasma membrane tyrosine kinase signaling and an increase in glucose metabolism. Finally, since Akt is known to effect glucose metabolism and the glucose transporter, GLUT1, we sought to determine the effects of reducing GLUT1 in breast cancer cells. Breast cancer cells with reduced GLUT1 have reduced glucose metabolism and reduced proliferation in vitro as well as reduced tumor growth in an athymic nude mouse breast cancer models.

15. SUBJECT TERMS
Akt, Trastuzumab, Herceptin, Apoptosis, GLUT1, ErbB2, Breast Cancer

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	30	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

INTRODUCTION

Trastuzumab is a drug which specifically targets HER2, an oncogene product overexpressed in a subset of breast cancers (1). Trastuzumab has been hailed for its specificity in targeting HER2 overexpressing cancer cells in contrast to standard chemotherapy regimens which target rapidly dividing cells and cause major side effects in treated patients. However, Trastuzumab only works in a third of patients with HER2 overexpressing breast cancer and has mild adverse side effects in 40% of patients and major adverse side effects in 5% of patients (2). Therefore, fully understanding the biology of Trastuzumab is important in order to identify patients who will respond to Trastuzumab therapy and to design combinations of therapies that will result in more patients responding. The originally proposed study sought to understand one of the signaling pathways affected by cells treated with Trastuzumab: the Akt pathway, which is known to mediate cell survival (3-5). Trastuzumab has been shown to cause cell cycle arrest in HER2 overexpressing cells (6) and sometimes induce apoptosis (6, 7). However, HER2 overexpressing cells can become resistant to Trastuzumab if Akt is not downregulated (8, 9). The originally approved study sought to understand whether downregulation of Akt by Trastuzumab induces apoptosis, and if so, which molecules downstream of Akt action are necessary targets of Trastuzumab action.

The original statement of work was modified when the results of task 1 and task 2 showed little promise (as described below and in the first annual summary) and two new tasks were approved in January 2008 which aimed to evaluate the role of Akt in ErbB2-mediated tumorigenesis. Akt has been shown to be a critical mediator of ErbB2-induced tumorigenesis (17, 18) so we overexpressed constitutively active Akt1 in the context of mammary tumors induced by ErbB2 in mice (task 3) and demonstrated that overexpression of Akt1 decreases the latency of ErbB2 tumorigenesis and also significantly alters the signaling pathways and metabolism of these tumors. These experiments have been published (19). To further evaluate the role of Akt in ErbB2 tumorigenesis, we sought to reduce Akt in cell lines using shRNA (task 4), but several attempts to reduce Akt activation by shRNA were unsuccessful and are described below. One of the molecules regulated by Akt is GLUT1 (13, 14), and we were successful in reducing GLUT1 using shRNA. While we were unable to reduce activation of Akt itself, reduction of GLUT1 in mammary carcinoma cells reduced glucose metabolism, reduced proliferation and growth in soft agar and inhibited the initial tumor progression of tumor cells transplanted into athymic nude mice.

BODY

Task 1 - Assess the effectiveness of trastuzumab at inducing death in cultured breast cancer cells expressing differing Akt1 molecules: wild type Akt1, constitutively activated mutant Akt1 (myr-Akt1), and kinase dead Akt1 as well as an empty vector control (months 1-9)

a. Obtain BT474 and SKBr3 human breast cancer cells from ATCC

b. Generate cell lines expressing wild type Akt1, constitutively activated mutant Akt1 (myr-Akt1), kinase dead Akt1 and empty vector control

c. Evaluate apoptosis following trastuzumab or vehicle treatment

Two human breast cancer cell lines, SKBr3 and BT474, both of which overexpress HER2 and are sensitive to Trastuzumab (9) were obtained from the ATCC. These cell lines were compared to two other human breast cancer cell lines which do not overexpress HER2: MDA231 and T47D. All cell lines were maintained in complete media (DMEM containing 10% FBS, 0.1 mM non-essential amino acids and 40 ng/ml insulin). Parallel cell monolayers were grown to near confluence and one dish was treated with 10 μg/ml Trastuzumab in complete media while the other was left untreated. Cells were incubated overnight and cell lysates were harvested 18 hours after treatment and then subjected to immunoblot analysis.

Previous reports have shown downregulation of HER2 after Trastuzumab treatment (10), but this has been disputed (6, 11). Our data indicates Trastuzumab does not regulate HER2 in the two cell lines which clearly overexpress HER2: SKBr3 and BT474 (Figure 1). MDA231 and T47D do not overexpress HER2 (Figure 1) and do not respond to trastuzumab. The decrease in HER2 expression after trastuzumab treatment in MDA231 and T47D cells (Figure 1) is likely inconsequential because HER2 is not overexpressed and HER2 is not responsible for tumorigenesis in these cell lines. EGFR and HER3, two other members of the EGFR family were also examined, and were also unaffected by Trastuzumab treatment. Trastuzumab does inhibit activation of Akt as seen by a decrease in Akt phosphorylation at Serine 473 after trastuzumab treatment in the two HER2

overexpressing cell lines, but not in the other two cell lines (Figure 1). Total levels of Akt and PTEN, a negative regulator of Akt activation, are not affected by trastuzumab treatment (Figure 1). This supports the hypothesis that downregulation of Akt by Trastuzumab in HER2 overexpressing cells could lead to activation of pro-apoptotic molecules normally regulated by Akt. Apoptosis was examined by probing for PARP, a substrate of caspases and thus a marker of apoptosis. BT474 cells demonstrate an increase in apoptosis (marked by increased level of cleaved PARP) after trastuzumab treatment, but trastuzumab treatment does not lead to apoptosis in SKBr3 cells (Figure 1). Thus, downregulation of Akt activation by trastuzumab in SKBr3 cells is not sufficient to cause apoptosis, but trastuzumab does cause an increase in apoptosis in BT474 cells.

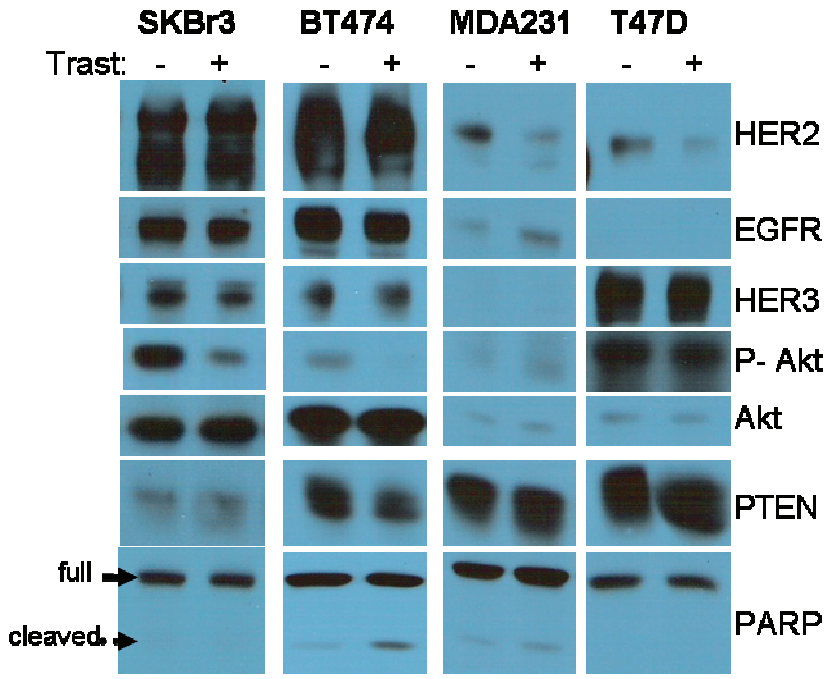


Figure 1 – The Effect of Trastuzumab on Akt Signaling, the EGFR Family and Apoptosis in Human Breast Cancer Cells.
Four different human breast cancer cell lines (SKBr3, BT474, MDA231 and T47D were cultured with or without 10 ug/ml Trastuzumab (+/- Trast) for 18 hours before harvesting whole cell lysates. Lysates were subjected to SDS-PAGE and immunoblot analysis with antibodies detecting HER2, EGFR, HER3, phosphorylated-Ser473-Akt (P-Akt), total Akt (Akt), PTEN, and PARP. The PARP antibody detects full length PARP and cleaved PARP which are indicated with arrows.

The lack of induction of apoptosis in SKBr3 cells and the modest induction of apoptosis in BT474 cells after Trastuzumab treatment indicate that apoptosis may not be a major effect of Trastuzumab, at least in cultured cell. Perhaps Trastuzumab induction of cell cycle arrest is more important to its anti-tumor effects (11) than induction of apoptosis. Alternatively, apoptosis may occur in vivo, in the context of an immune system and a tumor microenvironment, but not in cell culture. The second part of this aim sought to examine whether constitutively active Myr-Akt1 could rescue Trastuzumab induction of apoptosis, but this was not examined since Trastuzumab failed to induce apoptosis robustly. Other groups have demonstrated that activation of Akt causes Trastuzumab resistance (11, 12), but this is likely via affects other than apoptosis.

Task 2 - Identify molecules downstream of Akt1 inactivation essential for trastuzumab-mediated apoptosis. (months 9-30)

a. Identify which Akt substrates are activated by trastuzumab in wild type breast cancer cells: caspase 9, BAD, IKK, p53, SAPK pathway members and FOXO family members.

Lysates of cells treated 18 hours with 10 µg/ml Trastuzumab or lysates from untreated cells were used to examine the status of a few pro-apoptotic Akt substrates. FKHR is a transcription factor which is responsible for transcription of genes involved in cell cycle arrest and apoptosis (4). Akt phosphorylation of FKHR causes its transport out of the nucleus which attenuates transcription of pro-apoptotic and cell cycle arrest genes. Therefore, the phosphorylation status of FKHR was examined by immunoblot using a phospho-specific antibody which recognizes FKHR which has been phosphorylated by Akt. Trastuzumab down-regulation of Akt would be expected to cause a decrease in FKHR phosphorylation if FKHR is indeed a substrate in HER2 overexpressing breast cancer cells. However, Trastuzumab caused no difference in the phosphorylation of FKHR (Figure 2A). FKHR subcellular localization in BT474 cells was also examined before and after trastuzumab treatment, but FKHR appeared to be mostly nuclear (in the same fraction enriched for Lamin B), regardless of treatment (Figure 2B). Bad is another pro-apoptotic substrate of Akt and is eventually degraded

after phosphorylation by Akt (3). Bad was also evaluated by immunoblot, but no differences were detected between control lysates and lysates from Trastuzumab treated cells (Figure 2A and data not shown).

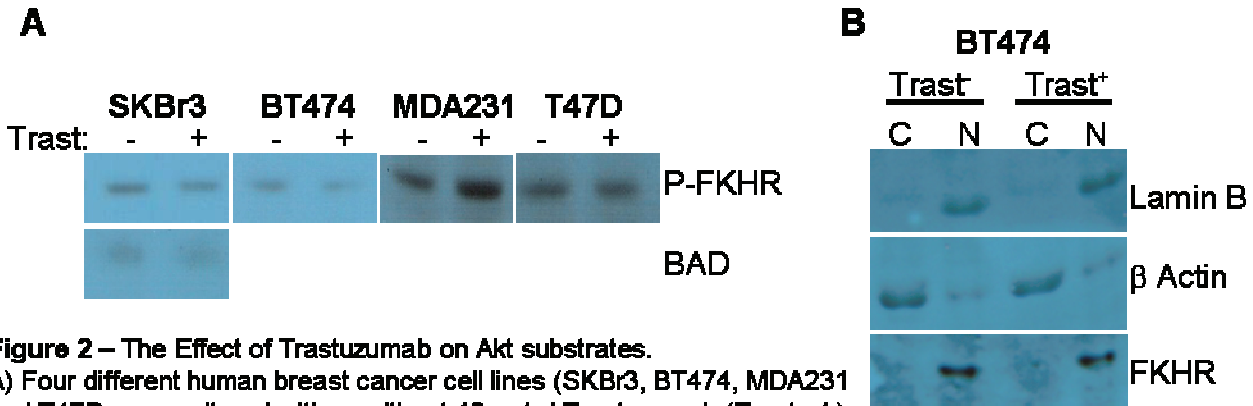


Figure 2 – The Effect of Trastuzumab on Akt substrates.
A) Four different human breast cancer cell lines (SKBr3, BT474, MDA231 and T47D were cultured with or without 10 ug/ml Trastuzumab (Trast +/-) for 18 hours before harvesting whole cell lysates. Lysates were subjected to SDS-PAGE and immunoblot analysis with antibodies detecting phosphorylated-FKHR (P-FKHR) and BAD. B) BT474 cells were cultured with or without 10 ug/ml Trastuzumab ($Trast^+$/$Trast^-$) for 18 hours before cytoplasmic-enriched and nuclear-enriched fractions were generated. Immunoblot analysis was performed with an antibody against Lamin B (nuclear marker), β-Actin (cytoplasmic marker) and FKHR to determine localizatioin of FKHR.

Trastuzumab was not efficiently inducing apoptosis in HER2 overexpressing breast cancer cells and the few pro-apoptotic substrates of Akt which were examined were also not being affected by Trastuzumab. Therefore, studies of Trastuzumab induction of apoptosis and further identification of pro-apoptotic Akt substrates downstream of Trastuzumab treatment were halted. However, identification of other important downstream affects of Akt inactivation following Trastuzumab treatment were pursued.

Akt has been shown to induce aerobic glycolysis in models of hematopoetic malignancies, partly via activation of the GLUT1 glucose transporter (13, 14). Additionally, it has been shown that HER2 overexpressing cancers stabilize HIF (hypoxia inducible factor) even in the absence of hypoxia, and HIF activates GLUT1 transcription (15, 16). Therefore, we wanted to test whether Trastuzumab had an affect on GLUT1 expression in both normoxic conditions and in the $CoCl_2$ model of hypoxia which stabilizes the HIF complex similar to hypoxia. BT474 were left untreated, treated with 100 µM $CoCl_2$, treated with 10 µg/ml Trastuzumab, or treated with both 100 µM $CoCl_2$ and 10 µg/ml Trastuzumab and incubated overnight. $CoCl_2$ clearly activated GLUT1 expression (Figure 3A, compare lane 1 to lane 3) while Trastuzumab alone slightly decreased GLUT1 expression (Figure 3A, compare lane 1 to lane 2). Additionally, Trastuzumab attenuated the $CoCl_2$ activation of GLUT1 (Figure 3A, compare lane 3 to lane 4). To investigate whether the trastuzumab affect on GLUT1 expression could be though Akt, a PI3K inhibitor, LY294002 (LY), was used to investigate whether inhibition of PI3K and Akt would recapitulate the results observed with Trastuzumab. SKBr3 and BT474 cells were left untreated or treated with 25 µM LY, 10 µg/ml Trastuzumab, 100 µM $CoCl_2$, both 25 µM LY and 100 µM $CoCl_2$, or both 10 µg/ml Trastuzumab and 100 µM $CoCl_2$ and incubated overnight. In both cell types, Akt was inactivated completely by LY and was partially inhibited by Trastuzumab while $CoCl_2$ treatment alone had no effect on Akt (Figure 3B and 3C). In SKBr3 cells, Trastuzumab and LY had no effect on basal GLUT1 levels, but both drugs inhibited the increase in GLUT1 seen after $CoCl_2$ treatment (Figure 3B). The inhibition was not complete because GLUT1 levels are still higher in drug plus $CoCl_2$ treated samples than the basal level seen without $CoCl_2$. This suggests that the increase in GLUT1 after $CoCl_2$ treatment requires PI3K or Akt, which are inhibited by LY and Trastuzumab. In BT474 cells, treatment with LY reduced the basal level of GLUT1 and also inhibited the $CoCl_2$ activation of GLUT1 (Figure 3C). Trastuzumab had a similar, but less pronounced affect as LY. The observation that LY strongly inhibits Akt activation and also strongly reduces GLUT1 (both with and without $CoCl_2$ treatment) and that Trastuzumab moderately inhibits Akt activation and moderately reduces GLUT1 activation by $CoCl_2$ suggests that Trastuzumab inactivation of Akt may be responsible for the decreased level of GLUT1. These results also indicate Trastuzumab may affect the metabolism of cancer cells in addition to its affects on cell cycle and cell signaling.

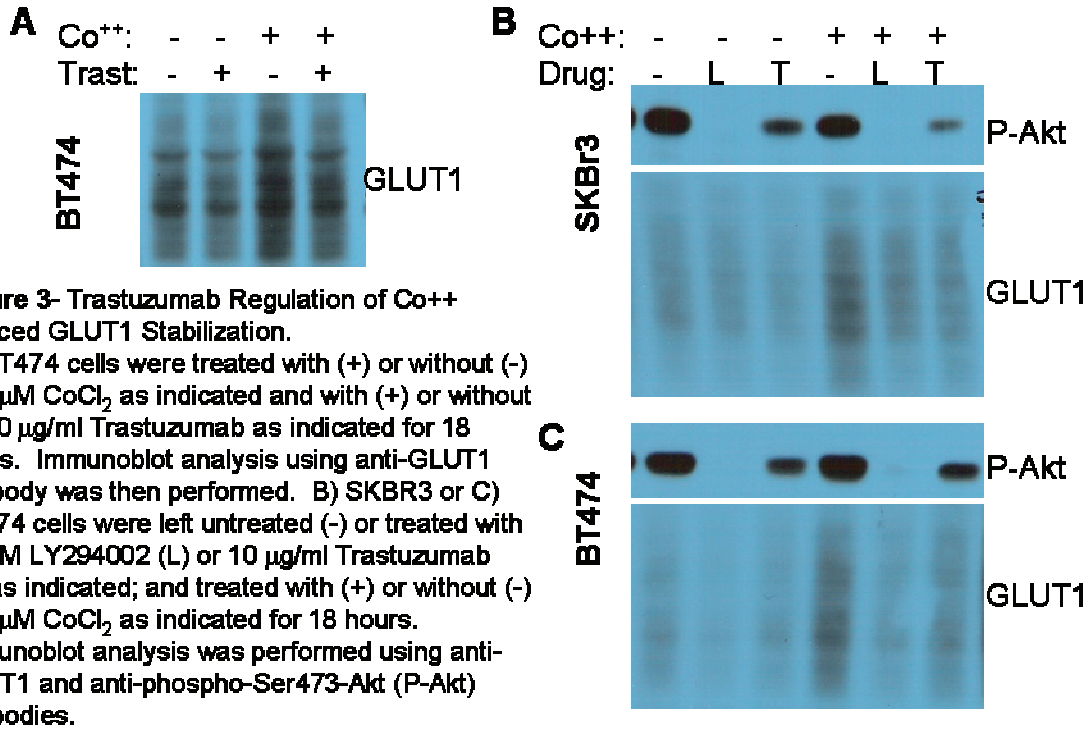


Figure 3- Trastuzumab Regulation of Co++ Induced GLUT1 Stabilization.
A) BT474 cells were treated with (+) or without (-) 100 μM $CoCl_2$ as indicated and with (+) or without (-) 10 μg/ml Trastuzumab as indicated for 18 hours. Immunoblot analysis using anti-GLUT1 antibody was then performed. B) SKBR3 or C) BT474 cells were left untreated (-) or treated with 25 μM LY294002 (L) or 10 μg/ml Trastuzumab (T) as indicated; and treated with (+) or without (-) 100 μM $CoCl_2$ as indicated for 18 hours. Immunoblot analysis was performed using anti-GLUT1 and anti-phospho-Ser473-Akt (P-Akt) antibodies.

January 2008 note: Trastuzumab was not effective at inducing apoptosis in SKBr3 cells and only weakly induced apoptosis in BT474 cells despite the effectiveness of trastuzumab at downregulating Akt activity in both ErbB2-overexpressing cell types. Additionally, downregulation of Akt substrates after trastuzumab treatment could not be proven. Therefore, a request to alter the statement of work was requested and the following two tasks which address the role of Akt1 in ErbB2-induced tumorigenesis were added and approved by GOR on 15 January 2008.

Task 3 – Determine the effect of activated Akt1 overexpression in ErbB2-overexpressing mouse mammary tumors (months 9-24)

a. Obtain six mammary tumors (and normal mammary gland controls) generated by MMTV-c-ErbB2 mice and six mammary tumors (and normal mammary gland controls) generated by bitransgenic MMTV-c-ErbB2, MMTV-myr-Akt1 mice

b. Divide tissue samples and send a portion for histologic sectioning and staining and lyse the remaining portion for immunoblot analysis

c. Perform immunoblot analysis comparing tumors from c-ErbB2 mice to bitransgenic c-ErbB2, myr-Akt1 mice as well as to normal mammary gland controls using the following antibodies (all commercially available): anti-HA antibody (to detect myr-Akt1 transgene), anti-ErbB2, anti-ErbB3, anti-ErbB4, anti-EGFR, anti-phospho-ErbB2 (Tyr877), anti-phospho-ErbB2 (Tyr1248), anti-phospho-ErbB3 (Tyr1289), anti-Rb, anti-phospho-Rb (Ser780), anti-Akt, anti-phospho-Akt (Ser473), anti-Src, anti-phospho-Src (Tyr416), 4G10 anti-phospho-tyrosine antibody, p15, p27 and anti-cyclin D1, and anti-ERK1 and anti-GLUT1.

d. Examine the histology of all tumors by hematoxylin & eosin staining and determine rates of apoptosis by anti-cleaved caspase 3 immunohistochemsitry

All of the objectives for this task have been completed and have been published. Please see the appended article for full description (19).

Task 4 – Evaluate tumorigenesis in a syngenic mouse mammary gland tumor cell transplant model by transplanting mouse mammary tumor cell lines with and without Akt1 expression as well as with and without Erk expression (months 24-36)

a. Obtain five cell lines generated from mammary tumors taken from MMTV-c-ErbB2 mice and determine the activation and expression level of Akt, ErbB2 and Erk.
b. Obtain shRNA molecules which target mouse Akt1 and shRNA molecules which target mouse Erk and evaluate their effectiveness at reducing expression of Akt1 or Erk1, 2 in cultured MMTV-c-ErbB2 tumor lines
c. Transplant mouse mammary tumor cells expressing control shRNA, Akt1 shRNA, Erk shRNA or Akt1 and Erk shRNA and determine rates of tumorigenesis and rates of tumor growth. This task will require 60 mice.
d. Using immunoblot analysis, evaluate the expression level and activation status of ErbB2, Akt, and Erk in tumors generated by tumor cell transplant.
e. Examine tumors, mammary glands and tumor cell injection sites by hemotoxylin & eosin staining

Expression (and phosphorylation) of EGFR family members, Akt, Erk and GLUT1 in cell lines established from ErbB2-induced tumors in mice. The laboratory of Ann Thor has established several cell lines from tumors derived from MMTV-c-ErbB2 mice (20) and the lysates for 5 of these cell lines (78617, 78622, 78717, 85815 & 85819) were examined by immunoblot (Figure 4). For controls, the lysates from an ErbB2 tumor is included in lane 1 (T) and lysates of HeLa cells in lane 2 of panel A and the lysates of Eph4 cells is included in lane 1 of panel B. The ErbB2 tumor and 78617, 85815 and 85819 cells express activated ErbB2 (P-ErbB2), ErbB2, activated Akt (P-Akt), EGFR, ErbB3 and activated Erk (P-Erk). The 78622 and 78717 cell lines lack the expression and activation of the EGFR family members as well as lack activation of Akt and Erk even though they express Akt and Erk (Figure 4). All cell lines and the tumor sample express GLUT1 with the exception of 85815 cells. This assortment of expression and activation of ErbB2, Akt, Erk and GLUT1 in this panel of cell lines provides the basis to either reduce expression of these molecules using shRNA or to overexpress these molecules. For example, 78617 cells contain activated ErbB2, Akt, Erk and GLUT1, so all these molecules can be potentially reduced by shRNA. 78717 cells lack ErbB2, activated Akt and Erk, so these molecules could be overexpressed in this cell line. Since 85815 cells have low levels of GLUT1, they provide a useful tool for the overexpression of GLUT1.

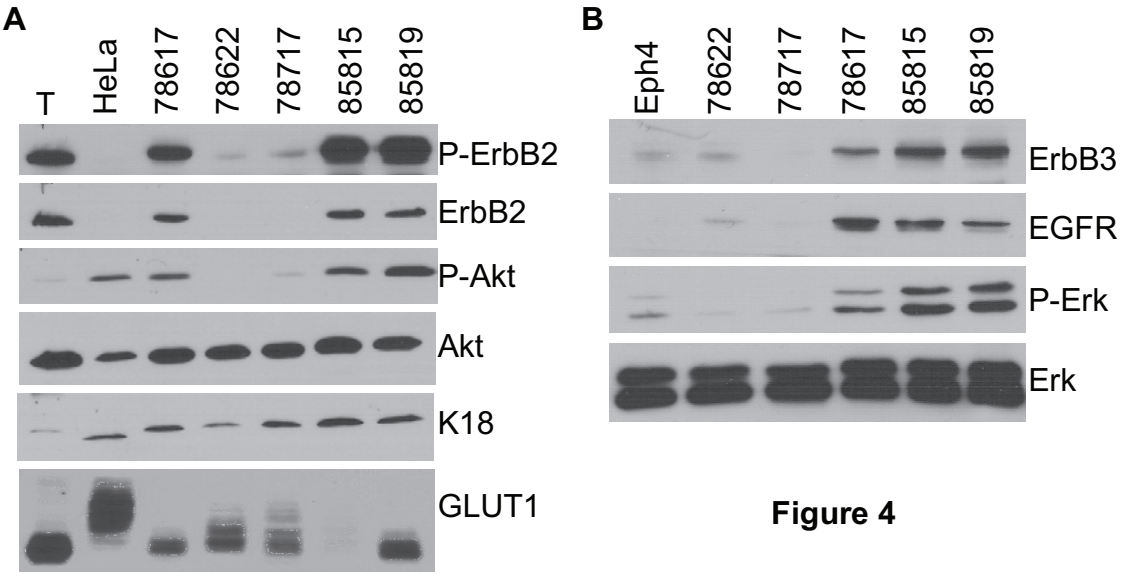


Figure 4

Evaluation of a doxycycline inducible shRNA construct for reduction of Akt1. A lentiviral-based plasmid (pTRIPZ) which has a short hairpin RNA that targets mouse Akt1 and a red fluorescent protein reporter (RFP) under control of a doxycycline inducible promoter was purchased from Open Biosystems. Lentiviral containing supernatants were created by co-transfecting pTRIPZ with pDelta8.9 (helper plasmid) and pVSVG (envelope plasmid) into 293T cells and harvesting/filtering the lentivirus containing supernatants at 48 hours. The supernatant was used to transduce 78617 and 85815 cells (which express Akt1 as shown above) as well as another mouse cell line, Met1.1, which is derived from a mammary tumor from a MMTV-polyoma middle T antigen mouse. Stable clones were established after selection in puromycin. Eight puromycin-resistant clones of each cell line were evaluated for their inducibility using the RFP reporter under control of the doxycycline-inducible promoter. Each clone was either left untreated (-Dox) or treated with 1 μg/ml doxyclycline (+Dox) for 24 hours and induction of RFP was evaluated using fluorescent microscopy (panel A). The three clones for each cell line that best induced RFP (red) are shown in panel A along with a blue nuclear counterstain (DAPI).

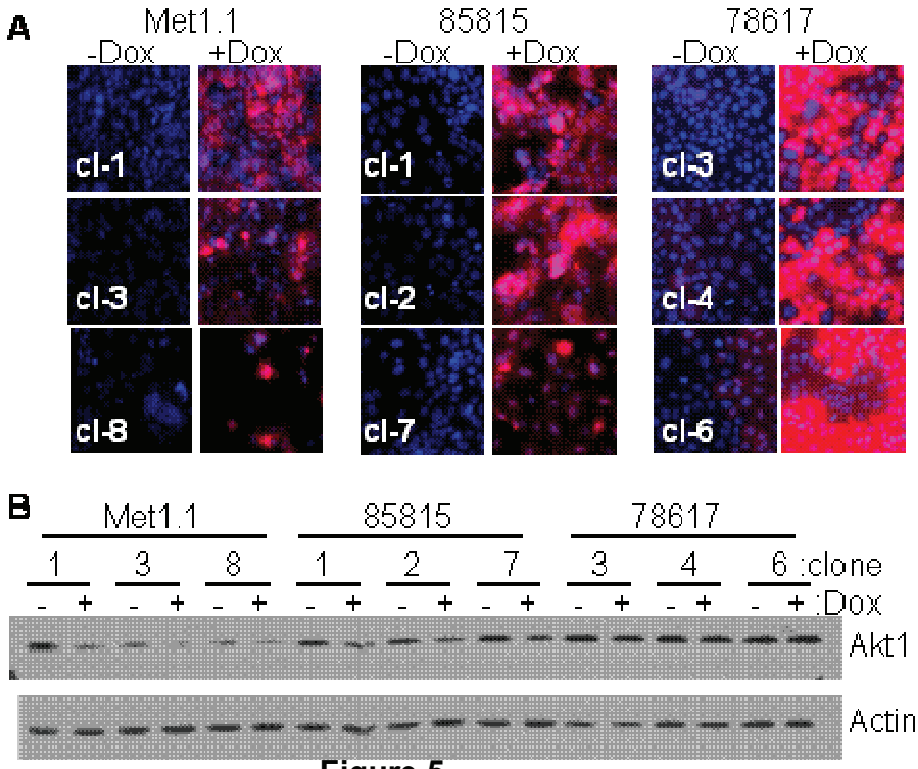


Figure 5

Doxycycline treatment should also induce the transcription of the Akt1 shRNA. To evaluate whether doxycycline treatment induced the Akt1 shRNA (and the subsequent reduction of Akt1), cell lines were treated -/+ doxycycline as indicated for 24 hours and the lysates were harvested and evaluated by immunoblot for Akt1 expression along with an Actin loading control (panel B). Clone 1 in Met1.1 cells demonstrates a doxycycline-dependent reduction of Akt1 while the other two clones in this cell line do not. Each of the three clones in 85815 cell demonstrate a modest reduction of Akt1 following the addition of doxycycline. None of the 786717 clones demonstrate a remarkable reduction of Akt1 at the 24 hour time point, though clone 3 may have a slight reduction. One reason for the poor reduction of Akt1 could be that 24 hours is not long enough to induce the shRNA and mediate the knockdown of Akt1 mRNA and protein. So, the best clone for each cell line was induced with with 1 µg/ml doxyclycline for 0, 1, 2 or 3 days and the lysates were collected and evaluated for Akt1 expression by immunoblot analysis (Figure 6). Clone 3 of 78617 cells and clone 1 of Met1.1 cells failed to demonstrate any reduction of Akt1 at any point during the 3 day experiment. Clone 2 of 85815 cells demonstrated a small decrease in Akt1 after 3 days of doxycycline induction (Figure 6).

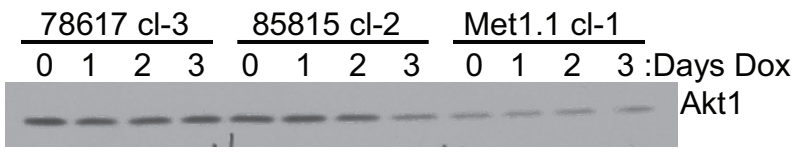


Figure 6

These numerous experiments suggested that the doxycycline inducible shRNA designed to reduce Akt1 was not effective, but one last experiment using the 85815 clone 2 cells was performed. These cells were treated with and without doxycycline for 2 days (as indicated), lysates were collected and immunoblot analysis was performed using an antibody that detects phosphorylated/activated Akt (P-Akt) as well as Akt1 (Figure 7). The shRNA failed to reduce Akt1 as well as Akt activation, so work with this doxycyline-inducible Akt1 shRNA lentivirus was discontinued. The goal of reducing activated Akt by reducing the major Akt isoform, Akt1, using this shRNA construct didn't appear to be effective despite months of troubleshooting.

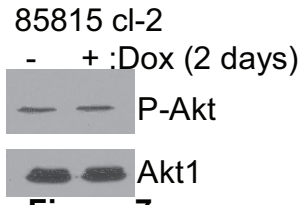


Figure 7

Evaluation of a panel of constitutively expressed shRNA constructs for reduction of Akt1.
Since the doxycycline-inducible Akt1 shRNA failed to work properly, we ordered 10 different shRNA constructs which were designed to target mouse Akt1 specifically and also ordered a control shRNA vector that targets GFP from Open Biosystems. These shRNA constructs are not inducible, nor do they contain the RFP reporter. Lentiviral containing supernatants were generated from the control GFP shRNA construct (G) as well as the ten Akt1 shRNA constructs (3, 4, 5, 6, 7, 34, 35, 36, 37 & 38) as described above and were used to transduce Met1.1 cells which express GFP-luciferase. Stable pools of cells were generated by selection in puromycin. Lysates were generated and evaluated by immunoblot analysis (Figure 8A). Immunoblot analyis using antibodies against Akt1 or total Akt demonstrated that shRNA vectors 35, 36, and 37 were the most effective at reducing Akt1 and total Akt. The control vector which targets GFP failed to reduce Akt1, but did reduce the level of GFP expressed in these cells (Figure 8A). After screening all eleven shRNA vectors, the

GFP shRNA vector and the sh38 vector were chosen as negative controls since they fail to reduce Akt while sh35 and sh37 were chosen because they effectively reduce Akt1 (Figure 8A). These four puromycin-resistant pools of cells were lysed and evaluated for their ability to reduce activated Akt as determined by immunoblot using an antibody which recognizes activated/ phosphorylated Akt (P-Akt) (Figure 8B). While shRNA 35 and 37 effectively reduced Akt1 and moderately reduced total Akt, they failed to reduce Akt phosphorylation (Figure 8B). This result means that any further data collection from these cell lines may be fruitless or confounding since reduction of Akt phosphorylation/activation and its subsequent effects on tumorigenesis was the main goal of task 4. Using these same shRNA constructs in 78617 cells failed to yield cells with reduced Akt activation.

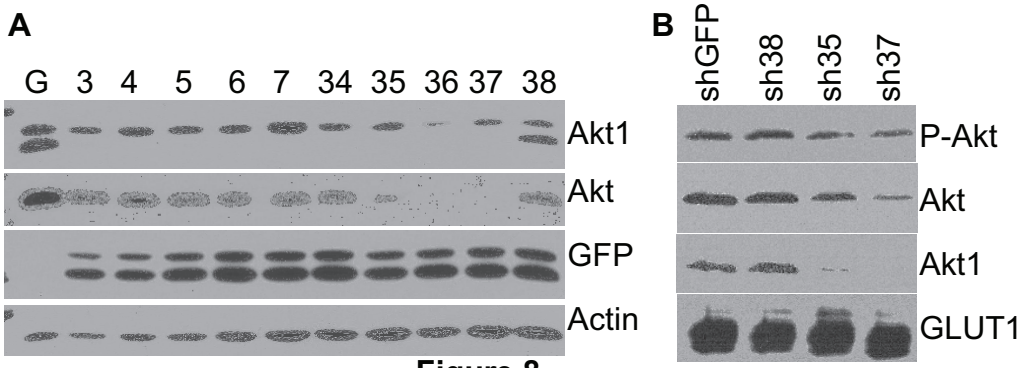


Figure 8

The overall goal of task 4 was to evaluate the role of Akt and Erk in ErbB2 tumorigenesis and multiple attempts to reduce Akt activation by shRNA were not effective (Figures 5-8) meaning the studies on tumorigenesis wouldn't be able to be performed adequately. While struggling to reduce Akt by shRNA, we were successful in reducing GLUT1, a glucose transporter which is known to be controlled (at least in part) by Akt (Figure 9). So, we pursued studies with these cell lines that have GLUT1 reduced by shRNA or have GLUT1 overexpressed and the results of these studies are discussed below.

Reduction of GLUT1 in 78617 GFP-luciferase cells reduces glucose consumption and lactate secretion

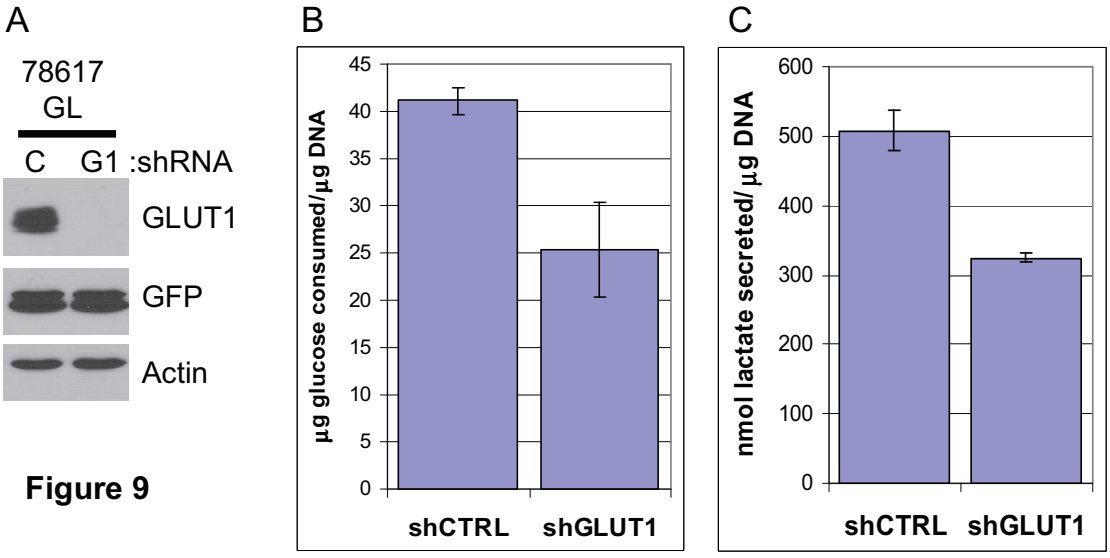


Figure 9

Figure 9A) 78617 GFP-Luciferase cells were stably transduced with lentivirus expressing control shRNA (C) or shRNA targeting GLUT1 (G1). A western blot demonstrates that the shRNA targeting GLUT1 reduces the amount of GLUT1 without effecting the levels of GFP-Luciferase or actin. B&C) 400,000 78617 GFP-Luciferase cells expressing control shRNA (shCTRL) or GLUT shRNA (shGLUT1) were seeded to quadruplicate wells of 12 well plates and incubated overnight. The conditioned media was collected at 24 hours and used to quanitate the amount of glucose consumed (panel B) and the amount of lactate secreted (panel C) as normalized to media incubated without cells. Glucose consumption and lactate secretion amounts are normalized to DNA content to adjust for any variance in cell number. This data is representative of two experiments and demonstrates that we can reduce GLUT1 and this results in a decrease in glucose consumption and secretion of lactate, a byproduct of glycolysis.

Reduction of GLUT1 in 78617 GFP-luciferase cells reduces glucose transport and proliferation

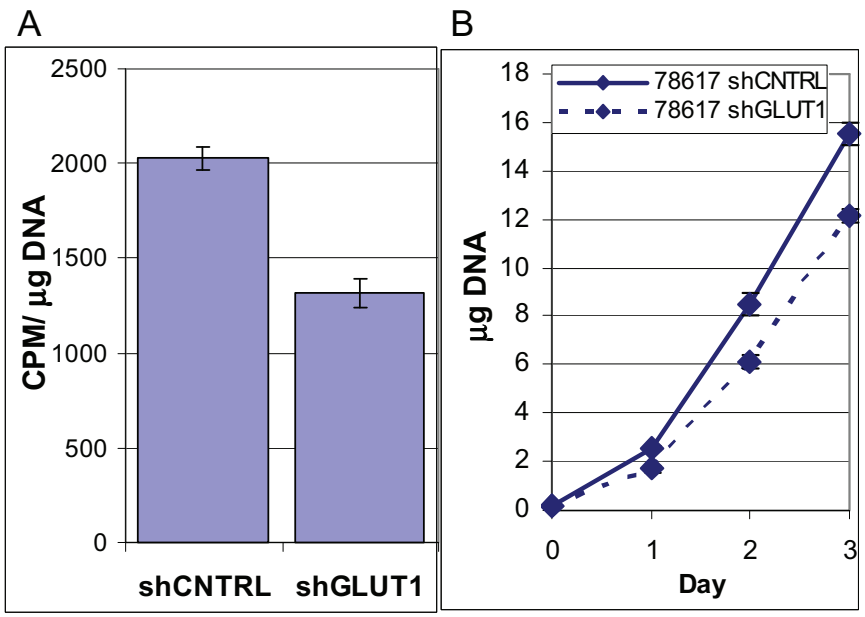


Figure 10 Figure 10A) 78617 GFP-Luciferase cells expressing control shRNA (shCNTRL) or shRNA targeting GLUT1 (shGLUT1) were seeded in quadruplicate wells of 24 well plates at a density of 200,000 cells per well and allowed to incubate overnight. The cells were washed with PBS and incubated 1 hour with DMEM lacking glucose followed by the addition of 2 µCi of ^{3}H-2-deoxyglucose which was incubated 15 minutes. Glucose transport was terminated by removing the media and washing the monolayers three times with ice cold PBS. Monolayers were lysed with NaOH and neutralized with equal volume HCl and counted by liquid scintillation. A parallel plate not incubated with ^{3}H-2-deoxyglucose was used to determine DNA concentration to which radioactivity counts (CPM) were normalized. This is representative of three experiments and demonstrates that shRNA against GLUT1 reduces glucose transport. Figure 10B) Proliferation of 78617 GFP-luciferase cells expressing control shRNA or GLUT1 shRNA was carried out by seeding 10,000 cells per well to quadruplicate wells of quadruplicate 24 well plates and lysing plates for DNA quantitation at 8 hours (day 0), 24 hours (day 1), 48 hours (day 2), and 72 hours (day 3). The graph indicates the increase in DNA content (~cell number) over the timecourse and that cells with reduced GLUT1 have a reduced rate of proliferation as compared to the control cells. This is representative of two experiments.

Reduction of GLUT1 in 78617 GFP-luciferase cells reduces growth in soft agar

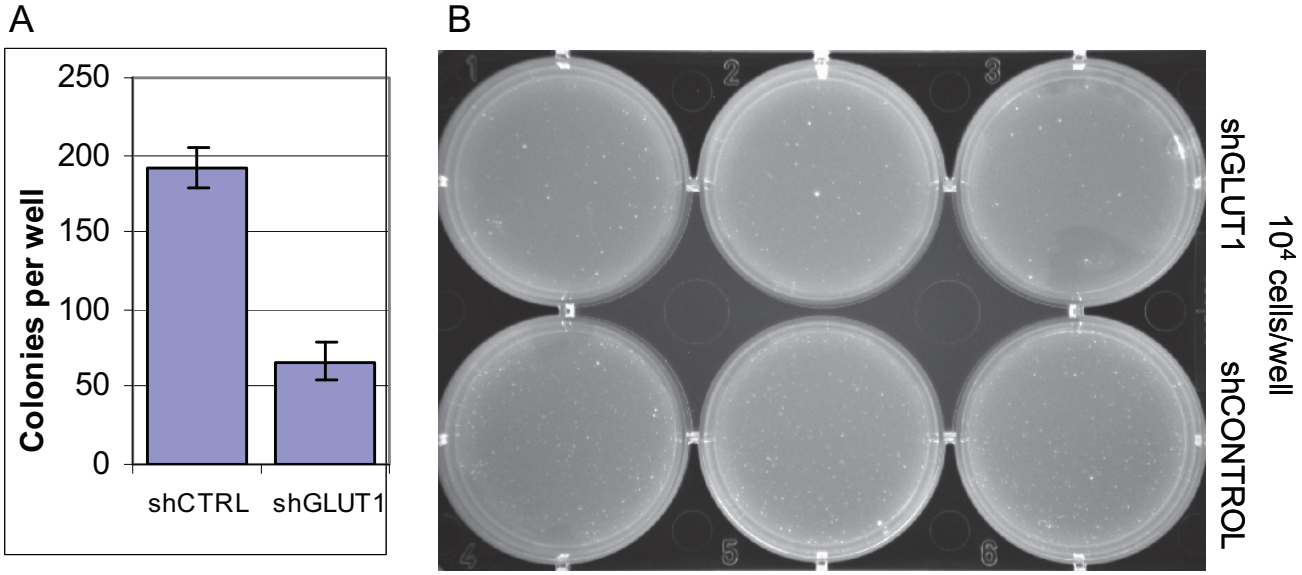


Figure 11 78617 GFP-luciferase cells expressing control shRNA or GLUT1 shRNA were seeded in 0.4% agar at 10,000 cells per well in triplicate to six well plates and incubated twenty days. Quantitation of the number of colonies per well is depicted in panel A and a picture of the plate is depicted in panel B of figure 11. These results are representatitive of two experiments. These data demonstrate that cells with reduced GLUT1 have reduced anchorage independent growth.

GLUT1 reduction in 85815 GFP-luciferase cells reduces glucose transport, but does not reduce proliferation

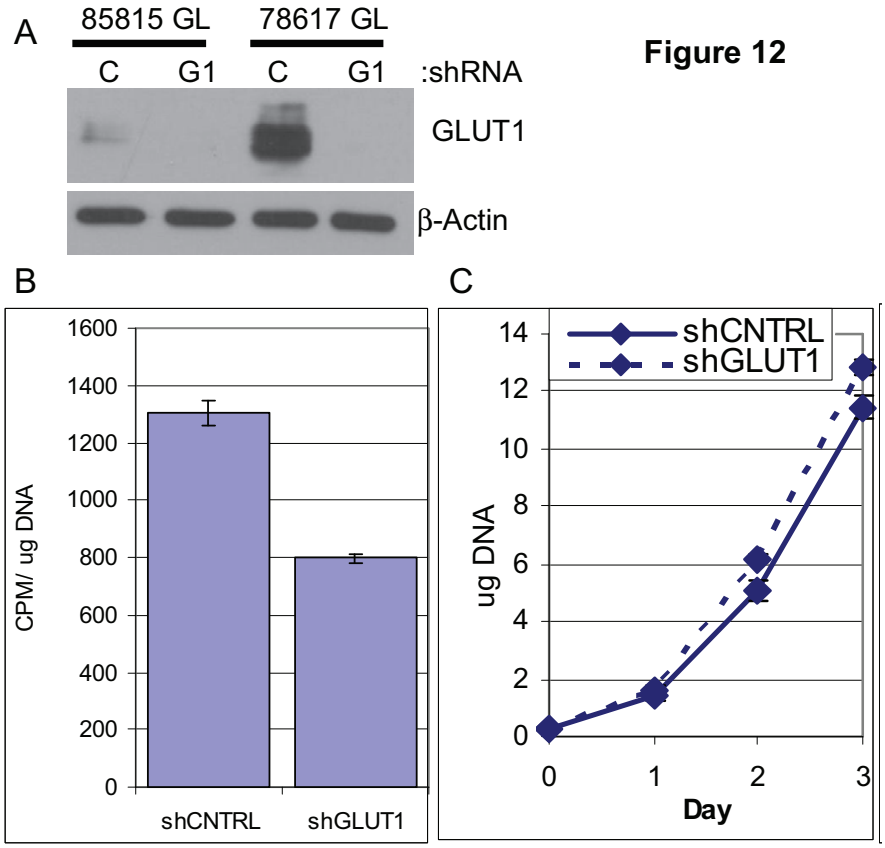


Figure 12

Figure 12A) GLUT1 was reduced in 85815 GFP-luciferase cells as explained in figure 9 and this immunoblot demonstrates the low basal level expression of GLUT1 in 85815 cells as compared to 78617 cells which robustly express GLUT1. Figure 12B) Uptake of of ^{3}H-2-deoxyglucose was examined as explained in figure 10a. 85815 cells expressing shGLUT1 have reduced ^{3}H-2-deoxyglucose transport as compared to control cells. This is representative of two experiments. Figure 12C) Cellular proliferation was measure as explained in figure 10b. 85815 GFP-luciferase cells expressing shGLUT1 do not exhibit impaired proliferation.

78617 GFP-luciferase cells expressing shRNA against GLUT1 have slower initial tumor progression than 78617 GFP-Luciferase cells expressing control shRNA.

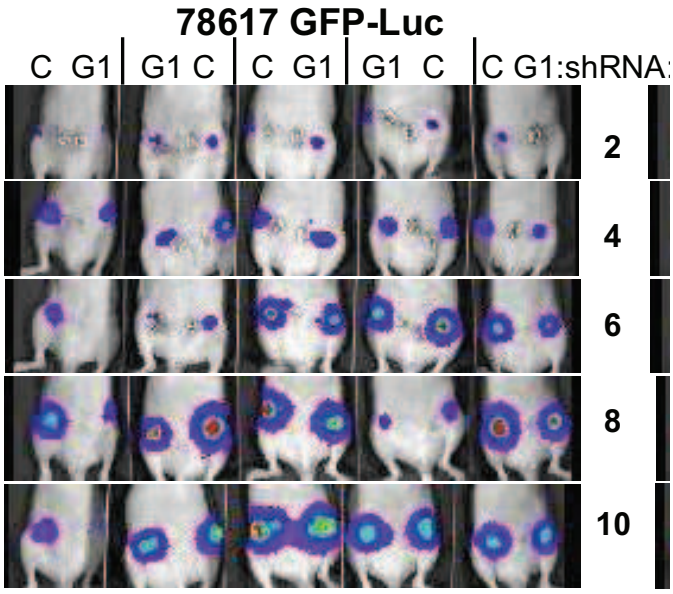


Figure 13

78617 GFP-luciferase cells expressing control shRNA (C) or GLUT1 shRNA (G1) were injected into contralateral #4 mammary fat pads of athymic nude mice (0.5 million cells in 20 µl 50% matrigel/50% PBS). Bioluminescence from the labeled tumor cells was detected on days 2, 4, 6, 8 and 10 after implantation using the IVIS bioimaging system (Figure 13). The pictures show abdominal region of the mice with a heat map depicting luciferase activity with purple being low activity and red being high activity. Each tumor can be seen to grow over time as indicated by increased heat map signal as the timecourse continues. This data is quantitated below in figure 14. The heat map qualitatively demonstrates that the tumors initiated from cells expressing GLUT1 shRNA have reduced growth as compared to the tumors initiated from control cells.

Quantitation of 78617 GFP-luciferase-derived tumors pictured in figure 13

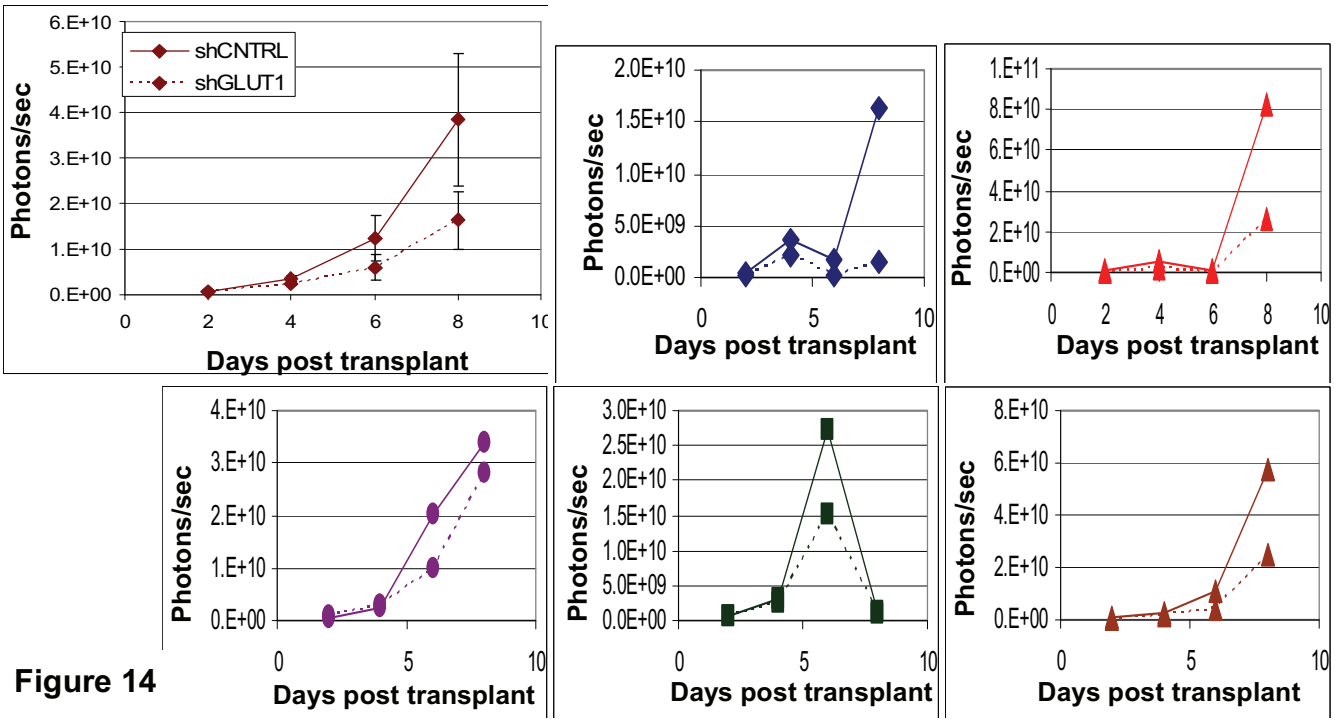


Figure 14

Figure 14) Each of the five smaller graphs represents the luciferase activity of a single mouse pictured in figure 13 as measured on days 2, 4, 6 and 8 while the larger graph in the upper left represents the average luciferase activity +/-SEM of all five mice. Each tumor derived from shGLUT1 cells (dashed lines) is smaller than the contralateral tumor derived from control shRNA expressing cells (solid lines) suggesting that GLUT1 is a critical mediator of tumor growth.

Tumors intiated from tumor cells expressing GLUT1 shRNA maintain decreased levels of GLUT1

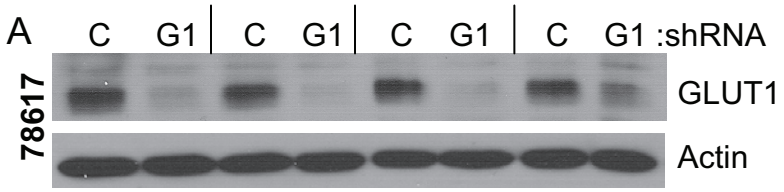


Figure 15

The animals described in figures 13 & 14 were sacrificed ten days after tumor cell injection and their tumors were removed. A portion of each tumor was lysed and analyzed for GLUT1 and actin expression by immunoblot. The tumors initiated from 78617 GFP-Luciferase cells expressing control shRNA have high levels of GLUT1 and the tumors initiated from cells expressing GLUT1 shRNA maintain low levels of GLUT1 indicating that the cells of these tumors maintain expression of the GLUT1 shRNA.

Overexpression of GLUT1 in 85815 cells does not increase glucose consumption or lactate secretion

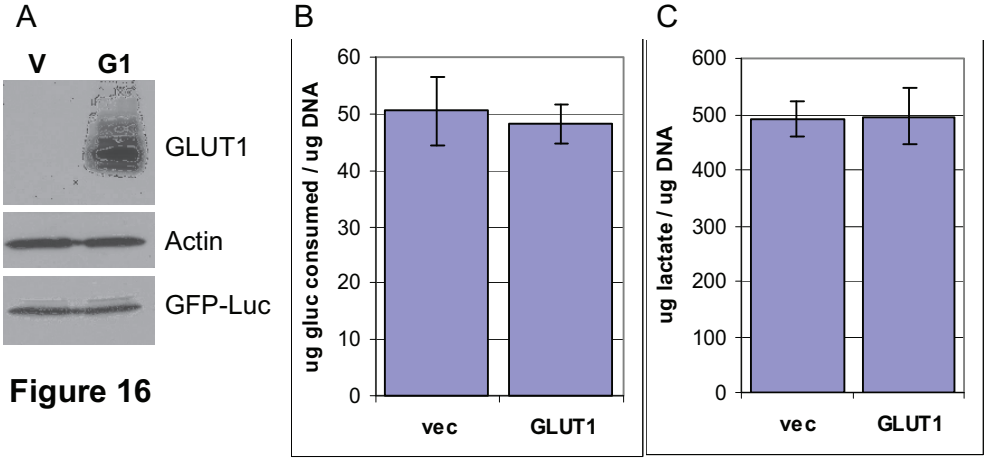


Figure 16

Figure 16A) 85815 GFP-luciferase cells (which have low levels of GLUT1) were transduced with a GLUT1 expressing retrovirus (G1) or an empty vector retrovirus (V) and this immunoblot demonstrates effective overexpression of GLUT1 without affecting the levels of actin or GFP-luciferase. These cells were evaluated for

glucose consumption (B) and lactate secretion (C) as described in figure 9, but overexpression of GLUT1 did not affect either of these activities. This data is representative of two experiments.

Overexpression of GLUT1 in 85815 GFP-luciferase cells increases glucose transport, but does not effect cellular proliferation

Figure 17

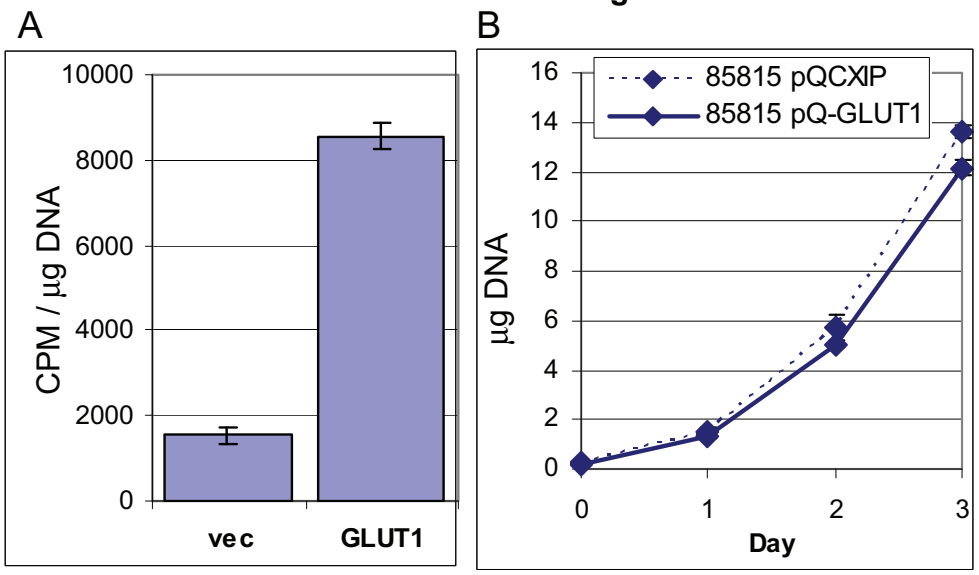
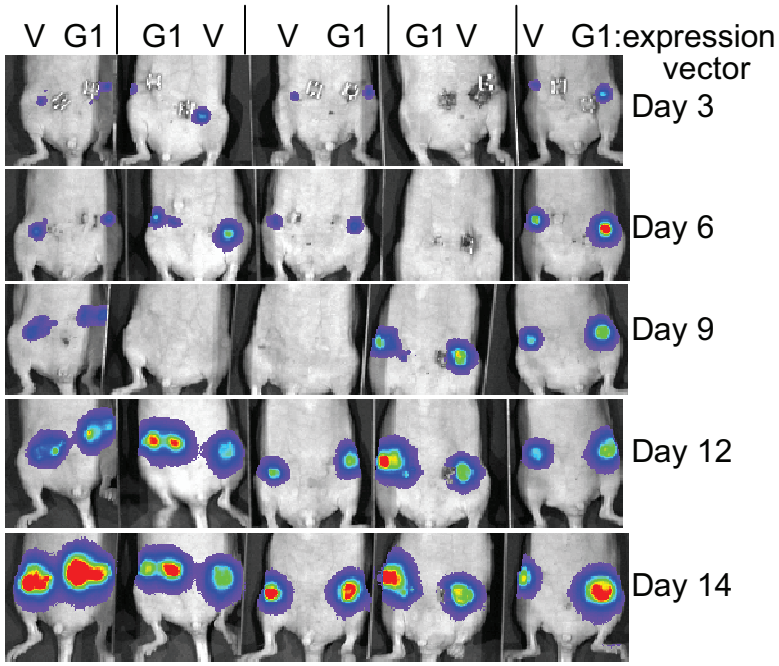


Figure 17A) 85815 GFP-luciferase cells overexpressing GLUT1 (GLUT1) or the empty vector control cells (vec) were analyzed for uptake of ^{3}H-2-deoxyglucose explained in figure 10. Cells overexpressing GLUT1 transport more ^{3}H-2-deoxyglucose than the control cells. B) Overexpression of GLUT1 does not effect proliferation. This assay was performed as described in figure 2b.

85815 GFP-luciferase tumor cells overexpressing GLUT1 have faster initial tumor progression than 85815 GFP-luciferase cells expressing empty vector



85815 GFP-luciferase cells expressing empty vector (V) or overexpressing GLUT1 (G1) were injected into the mammary fat pad of athymic nude mice as described in figure 13. The tumors were analyzed for bioluminescence on days 3, 6, 9, 12 and 14 post-transplant. The tumors initiated from cells overexpressing GLUT1 appear to have a faster rate of tumor growth as indicated by increased signal on the heat map as compared to the contralateral tumors initiated from control cells. This data is quantitated in figure 19

Figure 18

Quantitation of tumor growth initiated from 85815 cells shown in figure 18

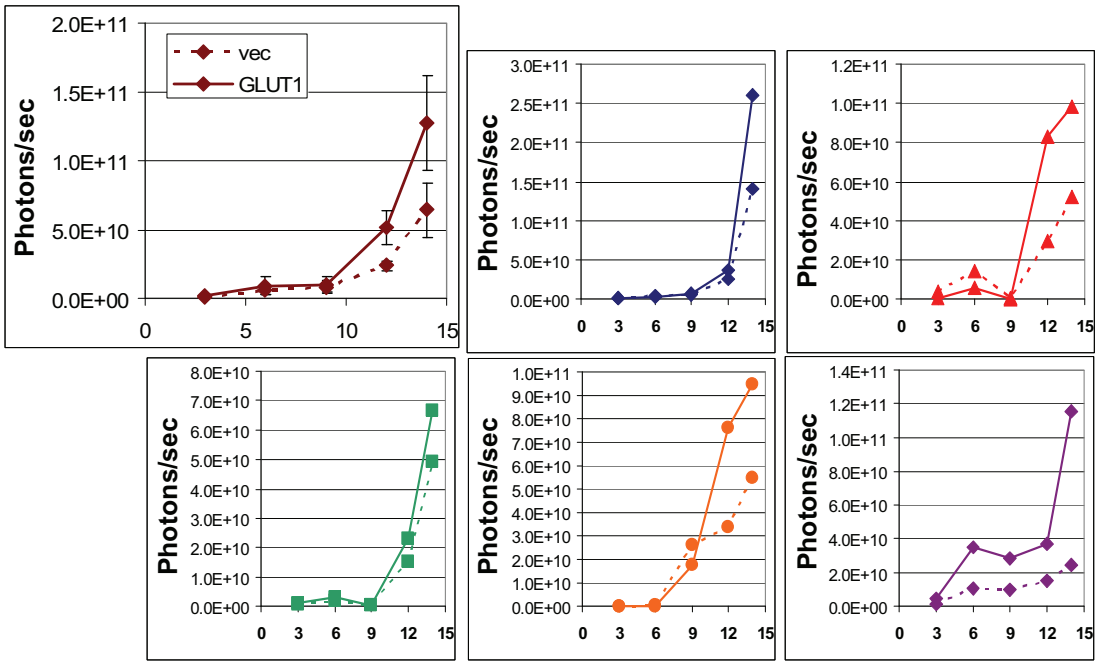


Figure 19: Each of the five smaller graphs represents the luciferase activity of a single mouse as measured on days 3, 6, 9, 12 and 14 while the larger graph in the upper left represents the average luciferase activity +/-SEM of all five mice. Each tumor derived from cells overexpressing GLUT1 (solid lines) is bigger than the contralateral tumor derived from control cells (dashed lines) at later time points.

KEY RESEARCH ACCOMPLISHMENTS

- Trastuzumab inhibits Akt activity in HER2 overexpressing breast cancer cell lines
- Inhibition of Akt activity by Trastuzumab fails to upregulate two pro-apoptotic substrates of Akt: FKHR and Bad
- Trastuzumab does not robustly induce apoptosis in vitro
- Trastuzumab inhibits the expression of GLUT1 and cobalt-induced increase in GLUT1 expression in a manner similar to inhibition of PI3K/Akt
- Overexpression of constitutively active Akt1 in the mammary tumors of MMTV-c-ErbB2 mice accelerates tumorigenesis
    - Tumors with activated Akt have a reduced requirement for a significant subset of plasma membrane tyrosine kinase signaling
    - Tumors with activated Akt have increased GLUT1 and increased glucose metabolism
- Reducing the expression of GLUT1 reduces the glucose metabolism of cultured tumor cells and also decreases proliferation, growth in soft agar and growth in an athymic nude mouse tumor model
- Overexpressing GLUT1 may increase glucose metabolism of breast cancer cells and increases their growth in an athymic nude mouse tumor model

REPORTABLE OUTCOMES

Publications during graduate school:

Young CD, Nolte EC, Lewis A, Serkova NJ, Anderson SM: **Activated Akt1 accelerates MMTV-c-ErbB2 mammary tumourigenesis in mice without activation of ErbB3**. *Breast Cancer Res* 2008, **10:**R70.

Young CD, Anderson SM. **Sugar and fat - that's where it's at: metabolic changes in tumors.** *Breast Cancer Res*. 2008,**10**(1):202.

Lambert JR, Young CD, Persons KS, Ray R. **Mechanistic and pharmacodynamic studies of a 25-hydroxyvitamin D3 derivative in prostate cancer cells.** *Biochem Biophys Res Commun.* 2007 Sep 14;361(1):189-95.

- All aspects of this work has been presented annually at the UCHSC Pathology Department Research in Progress Seminar as well as the UCHSC Pathology Department Grand Rounds
- "Activated Akt1 accelerates MMTV-c-ErbB2 mammary tumourigenesis in mice without activation of ErbB3" has been presented as a poster at a UCHSC cancer center conference as well as at the DOD BCRP Era of Hope meeting held in Baltimore in 2008
- The data regarding shRNA and overexpression of GLUT1 (figures 9-19 above) was submitted as an abstract for the 2009 American Association of Cancer Research (AACR) annual meeting and will presented as a poster at that meeting in April 2009 by CDY
    - Much of this data is being formally prepared for publication
- The thesis committee for CDY has approved his research (most of which is discussed above) and asked that he begin writing his dissertation which he is expected to defend in June 2009
- Numerous novel cell lines have been created:
    - 78617 cells expressing GFP-luciferase (as well as numerous shRNA vectors against GLUT1, GFP, and Akt)
    - 85815 cells expressing GFP-luciferase (as well as numerous shRNA vectors against GLUT1, GFP, and Akt)
    - Met1.1 cells expressing GFP-luciferase (as well as numerous shRNA vectors against GLUT1, GFP, and Akt)
    - 85815 cells expressing GFP-luciferase (as well as overexpressing empty vector or GLUT1)
- Steve Anderson (mentor to CDY) has included much of the data discussed in grant applications (to the DOD, Susan G Komen Foundation and the NIH) which propose to further evaluate the role of GLUT1 and other glucose transporters in breast cancer as well as broader aims of characterizing the metabolism of tumors

CONCLUSIONS

Using two HER2 overexpressing human breast cancer cell lines, SKBr3 and BT474, we demonstrated inactivation of Akt by Trastuzumab. However, inactivation of Akt failed to induce apoptosis in these cell lines. In agreement, Trastuzumab failed to upregulate two important pro-apoptotic substrates of Akt, Bad and FKHR. Additionally, the expression of HER2 and the other receptors of the EGFR family was similar before and after Trastuzumab treatment. Trastuzumab decreased the expression of the GLUT1 glucose transporter and also inhibited its cobalt-dependent increase in expression similar to inhibition of the PI3K/Akt pathway. This suggests the inhibition of Akt by Trastuzumab may be responsible for the reduced GLUT1 expression. Thus, while Trastuzumab does not seem to induce apoptosis, it may effect the metabolic needs of cancer cells via down regulation of GLUT1 and it does inhibit activation of Akt which others have shown causes growth arrest and sensitizes cancer cells to other drugs (11, 12).

Overexpression of constitutively active Akt in the mammary gland of MMTV-c-ErbB2 mice accelerates tumorigenesis. The accelerated tumorigenesis appeared to occur without some of the "classical" signaling that occurs downstream of ErbB2, such as activation of other EGFR members and Src. Thus, the bitransgenic mice overexpress ErbB2, but because they lack some of the signaling downstream of ErbB2, they may actually be resistant to trastuzumab because ErbB2 function may not be terribly important when Akt is activated by pathways other than ErbB2. The bitransgenic mice also overexpressed GLUT1 and had increased glucose metabolism which suggests another possible mechanism of increase tumorigenesis. Using shRNA in mammary tumor cells derived from ErbB2-induced tumors, we were able to reduce GLUT1 expression and this caused a decrease in glucose metabolism as well as growth tumor cell growth in vitro and and tumor growth in vivo. These results suggest that therapeutically targeting glucose metabolism in breast cancer is worth further investigation and may provide another molecularly targeted approach to cancer therapy.

REFERENCES

1 Menard,S., Pupa,S.M., Campiglio,M. and Tagliabue,E. Biologic and therapeutic role of HER2 in cancer, Oncogene, *22:* 6570-6578, 2003.

2 Vogel,C.L., Cobleigh,M.A., Tripathy,D., Gutheil,J.C., Harris,L.N., Fehrenbacher,L., Slamon,D.J., Murphy,M., Novotny,W.F., Burchmore,M., Shak,S., Stewart,S.J. and Press,M. Efficacy and Safety of Trastuzumab as a Single Agent in First-Line Treatment of HER2-Overexpressing Metastatic Breast Cancer, J Clin Oncol, *20:* 719-726, 2002.

3 Del Peso,L., Gonzalez-Garcia,M., Page,C., Herrera,R. and Nunez,G. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt, Science, *278:* 687-689, 1997.

4 Burgering,B.M.T. and Medema,R.H. Decisions on life and death: FOXO Forkhead transcription factors are in command when PKB/Akt is off duty, J Leukoc Biol, *73:* 689-701, 2003.

5 Gottlieb,T.M., Leal,J.F.M., Seger,R., Taya,Y. and Oren,M. Cross-talk between Akt, p53 and Mdm2: possible implications for the regulation of apoptosis, Oncogene, *21:* 1299-1303, 2002.

6 Sliwkowski,M.X., Lofgren,J.A., Lewis,G.D., Hotaling,T.E., Fendly,B.M. and Fox,J.A. Nonclinical studies addressing the mechanism of action of trastuzumab (Herceptin), Semin.Oncol., *26:* 60-70, 1999.

7 Mohsin,S.K., Weiss,H.L., Gutierrez,M.C., Chamness,G.C., Schiff,R., Digiovanna,M.P., Wang,C.X., Hilsenbeck,S.G., Osborne,C.K., Allred,D.C., Elledge,R. and Chang,J.C. Neoadjuvant trastuzumab induces apoptosis in primary breast cancers, J Clin.Oncol., *23:* 2460-2468, 2005.

8 Chan,C.T., Metz,M.Z. and Kane,S.E. Differential sensitivities of trastuzumab (Herceptin(R))-resistant human breast cancer cells to phosphoinositide-3 kinase (PI-3K) and epidermal growth factor receptor (EGFR) kinase inhibitors, Breast Cancer Res Treat., *91:* 187-201, 2005.

9 Nagata,Y., Lan,K.H., Zhou,X., Tan,M., Esteva,F.J., Sahin,A.A., Klos,K.S., Li,P., Monia,B.P. and Nguyen,N.T. PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients, Cancer Cell, *6:* 117-127, 2004.

10 Hudziak,R.M., Lewis,G.D., Winget,M., Fendly,B.M., Shepart,H.M. and Ullrich,A. p185^{HER2} monoclonal antibody has antiproliferative effects in vitro and sensitives human breast tumor cells to tumor necrosis factor, Mol.Cell.Biol., *9:* 1165-1172, 1989.

11 Yakes,F.M., Chinratanalab,W., Ritter,C.A., King,W., Seelig,S. and Arteaga,C.L. Herceptin-induced Inhibition of Phosphatidylinositol-3 Kinase and Akt Is Required for Antibody-mediated Effects on p27, Cyclin D1, and Antitumor Action, Can.Res., *62:* 4132-4141, 2002.

12 Le,X.F., Lammayot,A., Gold,D., Lu,Y., Mao,W., Chang,T., Patel,A., Mills,G.B. and Bast,R.C., Jr. Genes affecting the cell cycle, growth, maintenance, and drug sensitivity are preferentially regulated by anti-HER2 antibody through phosphatidylinositol 3-kinase-AKT signaling, J Biol Chem., *280:* 2092-2104, 2005.

13 Elstrom,R.L., Bauer,D.E., Buzzai,M., Karnauskas,R., Harris,M.H., Plas,D.R., Zhuang,H., Cinalli,R.M., Alavi,A., Rudin,C.M. and Thompson,C.B. Akt Stimulates Aerobic Glycolysis in Cancer Cells, Can.Res., *64:* 3892-3899, 2004.

14 Baldwin,S.A., Kan,O., Whetton,A.D., Martin,S., Fawcett,H.A.C., Flint,D.J. and Wilde,C.J. Regulation of the glucose transporter GLUT1 in mammalian cells, Biochemical Society Transactions, *22:* 814-817, 1994.

15 Laughner,E., Taghavi,P., Chiles,K., Mahon,P.C. and Semenza,G.L. HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression, Mol Cell Biol, *21:* 3995-4004, 2001.

16 Liu,L. and Simon,M.C. Regulation of transcription and translation by hypoxia, Cancer Biol Ther, *3:* 492 497, 2004.

17 Maroulakou IG, Oemler W, Naber SP, Tsichlis PN: Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice. *Cancer Res* 2007, 67:167-177.

18 Ju X, Katiyar S, Wang C, Liu M, Jiao X, Li S, Zhou J, Turner J, Lisanti MP, Russell RG, Mueller SC, Ojeifo J, Chen WS, Hay N, Pestell RG: Akt1 governs breast cancer progression in vivo. *Proc Natl Acad Sci U S A* 2007, 104:7438-7443.

19 Young CD, Nolte EC, Lewis A, Serkova NJ, Anderson SM: Activated Akt1 accelerates MMTV-c-ErbB2 mammary tumourigenesis in mice without activation of ErbB3. *Breast Cancer Res* 2008, 10**:**R70.

20 Kim, A., Liu, B., Ordonez-Ercan, D., Alvarez, K. M., Jones, L. D., McKimmey, C., Edgerton, S. M., Yang, X., and Thor, A. D. Functional interaction between mouse erbB3 and wild-type rat c-neu in transgenic mouse mammary tumor cells. Breast Cancer Res, *7*: R708-R718, 2005.

Research article

Open Access

Activated Akt1 accelerates MMTV-c-ErbB2 mammary tumourigenesis in mice without activation of ErbB3

Christian D Young[1,2], Erica C Nolte[1,2], Andrew Lewis[1], Natalie J Serkova[3] and Steven M Anderson[1,2]

[1]Department of Pathology, University of Colorado Denver, Research Complex I, South Tower, Mail Stop 8104, 12801 East 17thAvenue, Aurora, CO 80045, USA
[2]Program in Cancer Biology, University of Colorado Denver, Research Complex I, South Tower, Mail Stop 8104, 12801 East 17thAvenue, Aurora, CO 80045, USA
[3]Department of Anesthesiology, University of Colorado Denver, AO1, 12631 East 17thAvenue, Aurora, CO 80045, USA

Corresponding author: Steven M Anderson, steve.anderson@uchsc.edu

Received: 2 May 2008 Revisions requested: 27 Jun 2008 Revisions received: 29 Jul 2008 Accepted: 13 Aug 2008 Published: 13 Aug 2008

Breast Cancer Research 2008, **10**:R70 (doi:10.1186/bcr2132)
This article is online at: http://breast-cancer-research.com/content/10/4/R70

Abstract

Introduction ErbB2, a member of the epidermal growth factor receptor (EGFR) family, is overexpressed in 20% to 30% of human breast cancer cases and forms oncogenic signalling complexes when dimerised to ErbB3 or other EGFR family members.

Methods We crossed mouse mammary tumour virus (MMTV)-myr-Akt1 transgenic mice (which express constitutively active Akt1 in the mammary gland) with MMTV-c-ErbB2 transgenic mice to evaluate the role of Akt1 activation in ErbB2-induced mammary carcinoma using immunoblot analysis, magnetic resonance spectroscopy and histological analyses.

Results Bitransgenic MMTV-c-ErbB2, MMTV-myr-Akt1 mice develop mammary tumours twice as fast as MMTV-c-ErbB2 mice. The bitransgenic tumours were less organised, had more mitotic figures and fewer apoptotic cells. However, many bitransgenic tumours displayed areas of extensive necrosis compared with tumours from MMTV-c-ErbB2 mice. The two tumour types demonstrate dramatically different expression and activation of EGFR family members, as well as different metabolic profiles. c-ErbB2 tumours demonstrate overexpression of EGFR, ErbB2, ErbB3 and ErbB4, and activation/phosphorylation of both ErbB2 and ErbB3, underscoring the importance of the entire EGFR family in ErbB2-induced tumourigenesis. Tumours from bitransgenic mice overexpress the myr-Akt1 and ErbB2 transgenes, but there was dramatically less overexpression and phosphorylation of ErbB3, diminished phosphorylation of ErbB2, decreased level of EGFR protein and undetectable ErbB4 protein. There was also an observable attenuation in a subset of tyrosine-phosphorylated secondary signalling molecules in the bitransgenic tumours compared with c-ErbB2 tumours, but Erk was activated/phosphorylated in both tumour types. Finally, the bitransgenic tumours were metabolically more active as indicated by increased glucose transporter 1 (GLUT1) expression, elevated lactate production and decreased intracellular glucose (suggesting increased glycolysis).

Conclusion Expression of activated Akt1 in MMTV-c-ErbB2 mice accelerates tumourigenesis with a reduced requirement for signalling through the EGFR family, as well as a reduced requirement for a subset of downstream signaling molecules with a metabolic shift in the tumours from bitransgenic mice. The reduction in signalling downstream of ErbB2 when Akt is activated suggest a possible mechanism by which tumour cells can become resistant to ErbB2-targeted therapies, necessitating therapies that target oncogenic signalling events downstream of ErbB2.

ANOVA = analysis of variance; Akt1DD = constitutively active Akt1 mutant bearing two phospho-mimetic mutations; EGFR = epidermal growth factor receptor; ERK = extracellular-signal regulated kinase; FVB = inbred mouse strain which was exclusively used in this study; Gab2 = Grb2 associated binding protein 2; GLUT1 = glucose transporter 1; L2 = lactation day 2; HIF1 = Hypoxia inducible factor 1; MMTV = mouse mammary tumour virus; MRS = magnetic resonance spectroscopy; Myr-Akt1 = constitutively active Akt1 mutant bearing the myristolation sequence from Src; NMR = nuclear magnetic resonance; PI3K = phosphatidyl inositol-3 kinase; PTEN = phosphatase and tensin homologue deleted on chromosome 10; PVDF = poly-vinylidene difluoride; Rb = retinoblastoma; SD = standard deviation; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis; Shc = Src homology 2 domain-containing transforming protein C1; TMSP = trimethylsilyl propionic-2,2,3,3,-d4 acid.

Introduction

The ErbB2/Neu/HER2 oncogene is amplified and overexpressed in 20% to 30% of human breast cancer cases, and expression of ErbB2 is associated with aggressive metastatic tumour behaviour, decreased time to clinical relapse and poor prognosis [1,2]. The importance of c-ErbB2 in mammary tumourigenesis was further established through a study of transgenic mice that expressed either activated ErbB2 (Neu-NT) [3,4] or non-activated c-ErbB2 [5,6]. Activated ErbB2-induced tumours in male and female mice have an average time to tumour appearance of 114 and 89 days, respectively [3], while expression of non-activated c-ErbB2 in the mammary gland of transgenic mice resulted in tumour formation in female mice in 150 to 300 days, with the latency depending on the founder line examined [5,6]. The most extensively investigated transgenic line to date is the mouse mammary tumour virus (MMTV)-c-ErbB2 line 202 female mice in which adenocarcinomas appear with an average latency of 205 days [6]. Overexpression of c-ErbB2 mRNA and protein, elevated c-ErbB2 kinase activity and increased tyrosine phosphorylation of cellular proteins was observed in tumour tissue, but not in normal mammary tissue from the same mouse [6].

Protein levels of ErbB3 and tyrosine phosphorylation of ErbB3 are increased in mammary tumours from transgenic mice expressing activated ErbB2 (Neu-DL) [7], suggesting that ErbB2 and ErbB3 function as an oncogenic unit [8,9]. Holbro *et al.* [9] demonstrated that loss of either functional ErbB2 or ErbB3 results in a loss of tumour cell proliferation even though ErbB3 does not possess an active tyrosine kinase domain [10]. ErbB3 phosphorylation activates phosphatidylinositol 3-kinase (PI3K) and its downstream target, Akt, thus providing a possible mechanism for the requirement for both ErbB2 and ErbB3 in stimulating mammary tumourigenesis. This gives rise to the hypothesis that expression of activated Akt could compensate for the expression of ErbB3 in ErbB2-induced mammary tumours.

Our group and others have demonstrated that expression of activated Akt1 [11,12] or overexpression of non-activated Akt1 [13] can delay mammary gland involution. In spite of the fact that Akt was discovered as an oncogene which induces leukaemia [14], mammary tumours were not observed in these transgenic mice [11-13]. Hutchinson *et al.* demonstrated that activated Akt1 could accelerate mammary tumourigenesis in transgenic mice that express activated ErbB2 [15]. Similarly, deletion of one or both alleles of phosphatase and tensin homolog (PTEN), a negative regulator of Akt signalling, accelerates tumour induction in another ErbB2 mouse mammary tumour model [16]. In the current study, we demonstrate that transgenic expression of activated Akt1 can accelerate mammary tumourigenesis in the MMTV-c-ErbB2 mice. However, we observe a significant attenuation of tyrosine kinase signalling in tumours from the bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 animals compared with tumours from the MMTV-c-ErbB2 animals, particularly with regard to ErbB3 and Src. These results have implications for human ErbB2-positive tumours that may also have high levels of activated Akt, whether due to the loss of the tumour suppressor PTEN or mutations in either PI3K or Akt.

Materials and methods

Mice lines

The MMTV-c-ErbB2 (line 202) transgenic mice [6] were obtained from The Jackson Laboratory, Bar Harbor, ME. Details of the MMTV-myr-Akt1 mice have been previously described [11]. These two FVB-derived transgenic lines were crossed and progeny genotypes were determined by PCR analysis. Virgin MMTV-c-ErbB2 and bitransgenic MMTV-c-ErbB2, MMTV-myr-Akt1 female mice were palpated weekly to detect the presence of mammary tumours starting at 60 days of age. Tumours were excised when they reached 1 cm in diameter and 1 cm deep, and normal, non-tumourigenic mammary tissue was harvested from the same animal at the time of tumour harvest. All mice were maintained in the Center for Comparative Medicine at the University of Colorado Denver – Anshutz Medical Campus, an Association for Assessment and Accrediation of Laboratory Animal Care-approved facility, and used in accordance with Institutional Animal Care and Use Committee-approved protocols.

Isolation of tail DNA and genotyping by PCR

DNA was extracted from 1.5 cm sections of tail and genotyping was performed using previously described protocols [11]. Detection of the Myr-Akt1 transgene utilised a forward primer which anneals to the Akt1 sequence: (5'-GCCGCTACTATGCCATGAAGA-3') and a reverse primer which anneals to the HA (haemagglutin) epitope: (5'-GTAATCTGGAACATCGTATGGGTA-3'). Detection of the ErbB2 transgene utilised the forward primer (Neu-3) 5'-CGGAACCCACATCAGGCC-3' and the reverse primer (Neu-4) 5'-TTTCCTGCAGCAGCCTACGC-3' [17].

Mutation analysis of tumour *ERBB2* transgene

Total RNA isolated from tumour samples was subjected to single-strand cDNA synthesis using 2.5 μM random hexamers and 1 μg RNA. Amplification of the rat *ERBB2* gene, nucleotides 1492 to 2117, was performed using forward primer AB2913, 5'-CGGAACCCACATCAGGCC-3', and reverse primer AB1310, 5'-TTTCCTGCAGCAGCCTACGC-3', as previously described [18]. The PCR products were separated on a 2% agarose gel, the bands of interest (representing truncated *ERBB2*) were purified and then re-amplified using the same primers. Sequence analysis was conducted by the University of Colorado Cancer Center Sequencing Core using forward primer 1882, 5'-CACTACAAGGACTCGTCCT-3', and reverse primer 2133, 5'-CCAACGACCACCACTAAG-3'.

Histological analysis and mitotic index quantification

Dissected tumours and normal mammary tissue were fixed in 4% neutral buffer formalin, embedded in paraffin, sectioned (4 μm) and stained with haematoxylin and eosin. Histological sectioning and staining were performed by the Histology Service, Department of Pathology, University of Colorado School of Medicine. The mitotic index of tumours of each genotype was determined by counting the number of mitotic figures in 10 fields of view with a magnification of 500 and the data were presented as the mean of three tumours +/- standard deviation (SD).

Quantification of apoptotic cells

Detection of apoptotic cells was performed by immunohistochemical staining with anti-active caspase-3 antibody (Cell Signaling Technologies, Beverly, MA, USA). For antigen retrieval, slides in citrate buffer were heated in a microwave for 20 minutes and allowed to cool before blocking with 10% normal goat serum. Slides were incubated overnight at 4°C with anti-activated caspase-3 antibody at a dilution of 1:100. Non-specific peroxidase activity was quenched with 1% hydrogen peroxide followed by secondary antibody (goat anti-rabbit) then tertiary Vector ABC (Vector Laboratories, Burlingame, CA, USA). Colour development was achieved by incubation with DAB followed by counterstaining with Gill's Haematoxylin. Cell counts were performed on a minimum of five fields of view per slide from three mice (total cells counted ranged from 1800 to 2500 per mouse).

Immunoblot analysis

Protein was extracted from frozen tumour tissue and normal tissue by grinding them to a powder under liquid nitrogen, resuspended in Mammary Gland Lysis Buffer (50 mM Tris (2-Amino-2-(hydroxymethyl)-1,3-propanediol) pH 7.4, 150 mM sodium chloride, 2 mM EDTA, 50 mM sodium fluoride, 1% Triton X-100, 1% deoxycholic acid, 0.1% sodium dodecyl sulfate [SDS], 1 mM dithiothreitol, 5 mM sodium orthovanadate, 100 μg/ml phenylmethanesulphonylfluoride and a complete protease inhibitor cocktail (Roche Applied Sciences, Indianapolis, IN, USA)), followed by Dounce homogenisation. Lysates were clarified by centrifugation and protein concentrations determined using the Bradford assay (BioRad, Hercules, CA, USA). Equal amounts of total protein per lane (2 to 50 μg) were resolved on SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membrane (Immobilon-P, Millipore, Bedford, MA, USA) and immunoblotted with the desired antibody.

Anti-HA antibody was obtained from Roche Applied Sciences (Indianapolis, IN, USA). Anti-phospho-ErbB2 (Tyr877), anti-phospho-ErbB3 (Tyr1289), anti-phospho-retinoblastoma (Rb) (Ser780), anti-phospho-Akt (Ser473), anti-phospho-Src (Tyr416), anti-phospho-Gab2 (Tyr452), anti-phospho-Shc (Tyr313), anti-phospho-Shc (Tyr239/240), anti-Akt, anti-p15, anti-p27 and anti-cyclin D1 antibodies were obtained from Cell Signaling Technologies (Beverly, MA, USA). The anti-ErbB2, anti-ErbB3, anti-ErbB4, anti-EGFR, anti-Src, anti-Shc, anti-β-actin and anti-ERK1 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-phospho-ErbB2 (Tyr1248) was obtained from Abcam (Cambridge, MA, USA). The anti-Rb antibody was obtained from BD Pharmingen (San Jose, CA, USA). The anti-phospho-ERK antibody was obtained from Promega (Madison, WI, USA). The anti-Gab2 polyclonal antibody was generously supplied by Dr. Haihua Gu (University of Colorado, Denver, USA). The glucose transporter 1 (GLUT1) polyclonal antibody was generated by Global Peptide (Fort Collins, CO, USA) by immunising rabbits with a peptide corresponding to the C-terminus of the human/mouse GLUT1 sequence (KTPEELFHPLGADSQV) and affinity purifying the resulting IgG.

Metabolic profiling of tumours by magnetic resonance spectroscopy

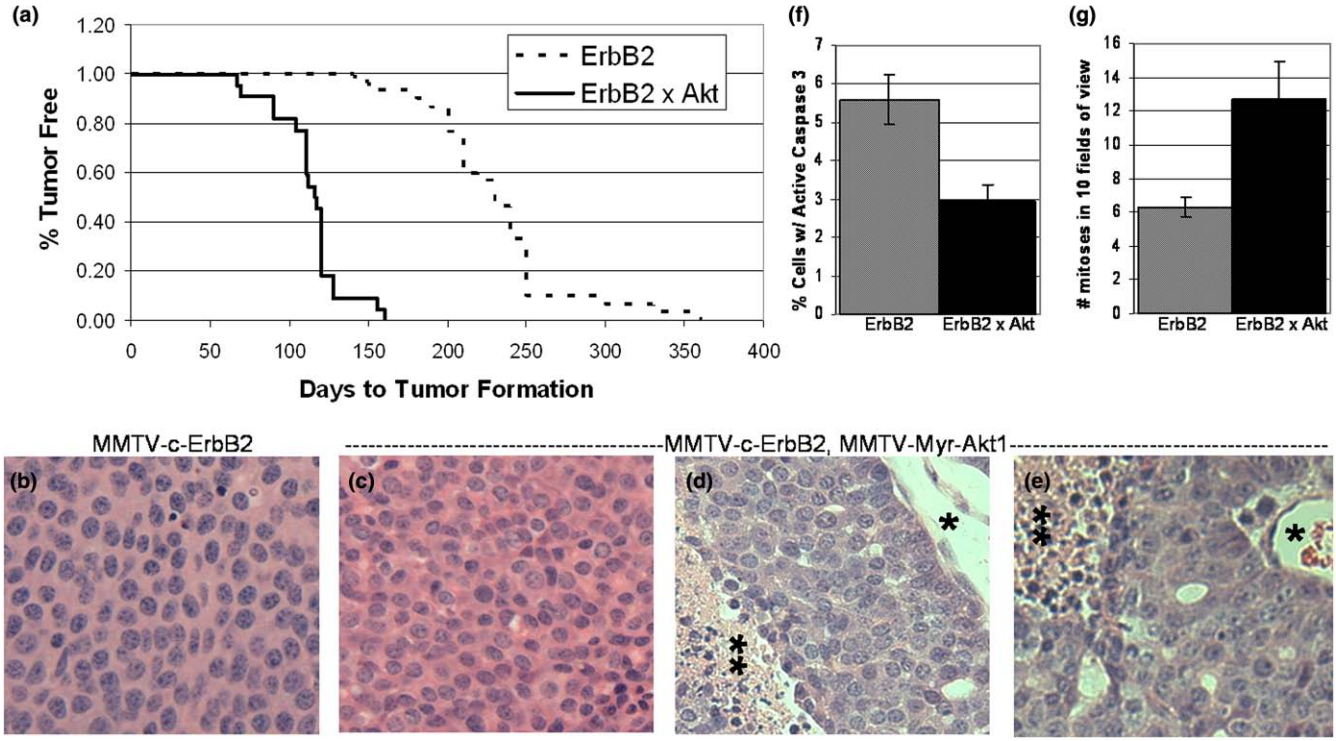
Snap-frozen tissues were ground into a powder under liquid nitrogen then homogenised by sonication in chloroform-methanol to precipitate proteins and separate aqueous and lipid-soluble metabolites as described [19]. The lyophilised aqueous and lipid extracts were dissolved in deuterated solvents and analysed using high-resolution ^{1}H-magnetic resonance spectroscopy (MRS) with the Bruker narrow-bore 500 MHz DRX system and an inverse TXI-5-mm probe (Bruker Biospin Inc., Fremont, CA, USA). The following ^{1}H-nuclear magnetic resonance (NMR) parameters with water suppression ('zgpr') were used: 500.12 MHz operating ^{1}H frequency; 40 accumulations; 0 dB power level; 55 dB irradiation power level; 7.5 μs pulse width; 12 ppm spectral width; and 12.8 second repetition time (fully relaxed). An external standard substance, trimethylsilyl propionic-2,2,3,3,-d_4 acid ([TMSP] 20 and 50 mM in heavy water) in a thin sealed glass capillary was placed into the NMR tubes during all experiments for metabolite quantification and as a ^{1}H chemical shift reference (at 0 ppm). After performing Fourier transformation, phase and base line corrections, each ^{1}H peak of corresponding metabolites was integrated using 1D WINNMR program (Bruker Biospin Inc., Fremont, CA, USA). The NMR peak assignment was confirmed by two-dimensional NMR spectra [19]. All quantitative data sets from ^{1}H-MRS are reported as mean ± SD ($n = 5$ for each group). The p values (from analysis of variance [ANOVA]) below 0.05 were considered as statistically significant.

Results

Activated myr-Akt1 accelerates mammary tumourigenesis in MMTV-c-ErbB2 transgenic mice

We have previously described the transgenic mice that express the activated myr-Akt1 transgene in the mammary gland which rarely develop tumours [11]. MMTV-c-ErbB2 (line 202) mice express the wild type rat *ERBB2* gene in the mammary gland and have been an extensively used model of mammary carcinomas [20-22]. To determine whether myr-Akt1 could accelerate mammary tumourigenesis, we crossed

Figure 1



Bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice have decreased mammary tumour latency, more aggressive tumour histology and decreased apoptosis compared with MMTV-c-ErbB2 mice. **(a)** Mammary tumour latency in bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice and MMTV-c-ErbB2 mice. Sixty days after birth, bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 and MMTV-c-ErbB2 mice were palpated weekly to monitor for the presence of mammary tumours. The graph shows the rate at which tumours were first detected for both genotypes. A total of 22 bitransgenic mice and 30 MMTV-c-ErbB2 mice were monitored and the graph shows the number of days to tumour detection versus the percentage of tumour-free mice. **(b-e)** Haematoxylin and eosin stained tumour sections. (b) Tumour derived from a MMTV-c-ErbB2 mouse. (c-e) Tumors derived from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice. (c) A bitransgenic tumour with histology similar to that of c-ErbB2 tumours. (d-e) Two different bitransgenic tumours demonstrating necrotic tumour tissue distal to a blood vessel (blood vessel marked with * and necrosis marker with **). ×200 original magnification. **(f)** Tumours from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice have less apoptosis than tumours from MMTV-c-ErbB2 mice. Apoptosis was quantitated by activated caspase-3 immunohistochemistry. The number of cells staining positively for activated caspase-3 was divided by the total number of cells counted to generate the apoptotic rate. **(g)** Tumours from bitransgenic mice have a higher proliferation rate than tumours from MMTV-c-ErbB2 mice. Proliferation rate was determined by counting the number of mitotic figures in 10 500× magnification fields of view and the data is presented as the mean +/- standard deviation for three tumours of each genotype.

MMTV-c-ErbB2 (line 202) mice [6] with MMTV-myr-Akt1 mice [11]. The MMTV-c-ErbB2 female mice developed single focal mammary tumours with a mean latency of 231 days (Figure 1a).

The bitransgenic MMTV-c-ErbB2, MMTV-myr-Akt1 mice developed single focal mammary tumours with a mean latency of 114 days, meaning the bitransgenic animals develop mammary tumours twice as fast as the MMTV-c-ErbB2 mice (Figure 1a). A total of 30 MMTV-c-ErbB2 mice and 22 bitransgenic mice were used to calculate mean tumour latency.

Tumours arising in the MMTV-c-ErbB2 mice usually display mutations in the wild type rat *c-ERBB2* transgene resulting in a constitutively activated form of ErbB2 [18]. One possible means by which expression of myr-Akt1 could accelerate tumourigenesis in the MMTV-c-ErbB2 mice would be to bypass the apparent requirement for mutation of rat *c-ERBB2*. However, analysis of the tumours that appeared in the bitransgenic mice indicated that these tumours also contain the activating mutations in the rat *c-ERBB2* allele (data not shown).

Tumour histology was evaluated using haematoxylin and eosin stained sections. The histology of the c-ErbB2 tumours was consistent with previous descriptions: they are solid tumours composed of uniformly sized and shaped cells with small stroma and no evidence of myoepithelial cells [6,23] (Figure 1b). Staining of tumours from the bitransgenic animals revealed two different types of tumours: those similar to c-ErbB2 tumours with a solid, uniform architecture (Figure 1c); and the second type of tumour demonstrated necrosis in areas of the tumour 10 to 20 cells away from vasculature, consistent with a tumour that outgrows its blood supply (Figure 1d, e). Very little necrosis is ever observed in tumours derived from MMTV-c-ErbB2 mice.

After initial detection, tumour volumes were determined by measuring the tumour dimensions with calipers to estimate tumour volume (volume = (length × width × width)/2). A comparison of tumour growth in mice of both genotypes revealed that tumour volume increased two to three times faster in the bitransgenic mice compared with tumours in the MMTV-c-ErbB2 transgenic mice (data not shown). The increased growth rate of tumours in the bitransgenic mice could result from either increased proliferation, decreased apoptosis or both. Measurement of the apoptotic rates in both tumour types by activated caspase-3 immunohistochemistry demonstrates that the tumours from the bitransgenic animals have an apoptotic rate half that of the tumours derived from the MMTV-c-ErbB2 animals (Figure 1f). The rate of proliferation, determined by quantitating the number of mitotic figures in tumour sections, demonstrated that tumours from bitransgenic animals had a higher proliferation rate than tumours from MMTV-c-ErbB2 animals (Figure 1g). Thus, the bitransgenic animals rapidly develop mammary tumours with a low rate of apoptosis and high rate of proliferation compared with the tumours from MMTV-c-ErbB2 animals, and at least half of these bitransgenic tumours exhibit extensive necrosis.

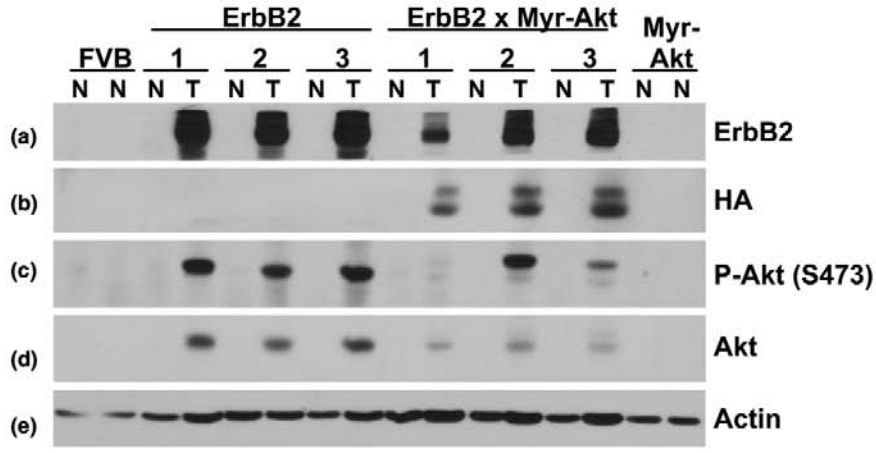
The expression of the transgenes was examined at the protein level in tumours (T) and normal mammary gland control (N) taken from tumour-bearing mice of both the MMTV-c-ErbB2 and MMTV-c-ErbB2, MMTV-myr-Akt1 genotypes. Mammary glands from FVB mice and MMTV-myr-Akt1 mice were used as controls. Immunoblotting with an anti-ErbB2 antibody demonstrated that the level of ErbB2 protein was dramatically increased in tumours of both origins compared with normal mammary tissue from the same mouse, from FVB mice or from myr-Akt1 transgenic mice (Figure 2a).

Expression of the HA-tagged myr-Akt1 transgene was only detected in tumour tissue from the bitransgenic animals (Figure 2b). The myr-Akt1 transgene in the bitransgenic tumours was phosphorylated at Ser473, indicating enzymatic activity, and can be distinguished from endogenous Akt because the myr-Akt1 transgene has a higher molecular weight (Figure 2c). Akt was also phosphorylated in c-ErbB2 tumours, consistent with previously published data [8] (Figure 2c). Immunoblot using anti-pan-Akt antibody demonstrates expression of endogenous Akt in both tumour types with the c-ErbB2 tumours expressing more Akt than the bitransgenic tumours (Figure 2d). An immunoblot with anti-β-actin antibody demonstrates equal sample loading (Figure 2e). All immunoblot data presented in the present study is representative of all tumour and normal gland pairs examined (n = 5 for c-ErbB2 and n = 7 for bitransgenic). The different levels of proteins expressed in tumour tissue versus normal tissue is probably due to both a difference in selective pressures brought on by tumourigenesis and a dramatic increase in epithelial cell content in tumour tissue: the virgin mammary gland is predominantly adipocytes and tumour tissue is predominantly epithelial in nature.

Diminished overexpression and activation of EGFR family members in tumours from bitransgenic animals

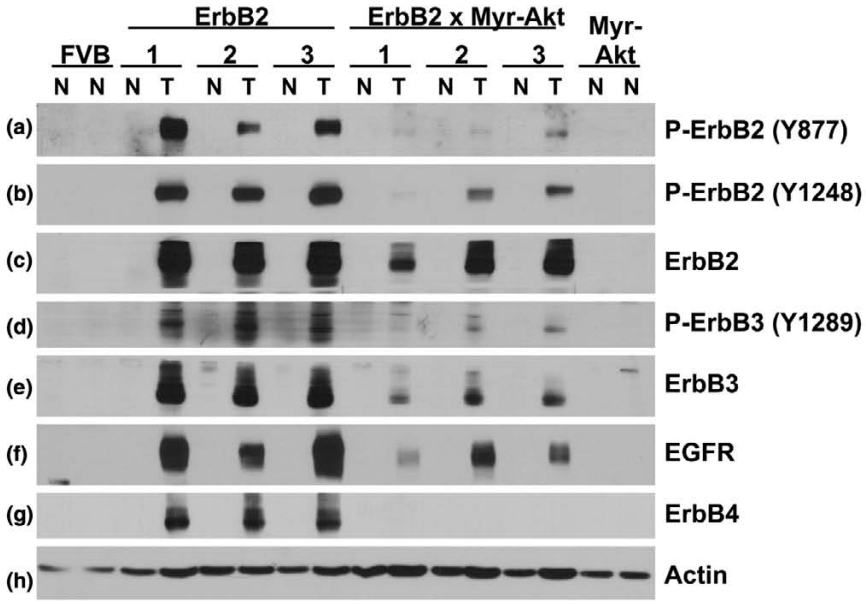
Mammary tumours from transgenic mice expressing activated mutants of ErbB2 also express elevated levels of total and tyrosine-phosphorylated ErbB2 and ErbB3 [7]. As previously shown in Figure 2, ErbB2 was increased in tumour lysates from both MMTV-c-ErbB2 and bitransgenic mice compared

Figure 2



Tumours from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice and MMTV-c-ErbB2 mice overexpress ErbB2 and phosphorylated Akt. Tumour (T) and normal mammary tissue (N) were harvested from tumour-bearing mice. Tumours from three different bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice and three different MMTV-c-ErbB2 mice are represented with normal mammary tissue from the same animal. Additionally, normal mammary tissue was collected from control FVB and MMTV-myr-Akt1 mice and analysed as a control. Equal amounts of total protein was loaded per lane to a 10% sodium dodecyl sulfate polyacrylamide gel, transferred to polyvinylidene difluoride and probed with the following antibodies: **(a)** anti-ErbB2; **(b)** anti-HA to detect the HA epitope-tagged myr-Akt1 transgene; **(c)** anti-phospho-Akt (Ser473); **(d)** anti-pan-Akt; and **(e)** anti-β-actin to demonstrate equal loading of the gel.

Figure 3



Expression and activation of EGF receptor tyrosine kinase family members is decreased in tumours from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice compared with tumours from MMTV-c-ErbB2 mice. Lysates from tumour (T) and normal mammary tissue (N) were used for immunoblot analysis using the following antibodies: **(a)** anti-phospho-ErbB2 (Tyr877); **(b)** anti-phospho-ErbB2 (Tyr1248); **(c)** anti-ErbB2; **(d)** anti-phospho-ErbB3 (Tyr1289); **(e)** anti-ErbB3; **(f)** anti-EGFR; **(g)** anti-ErbB4; and **(h)** anti-β-actin to demonstrate equal loading of the gel.

with normal tissue (Figure 3c). The extent of activating phosphorylation of ErbB2 was lower in the bitransgenic tumours than in the c-ErbB2 tumours, demonstrated by using phosphospecific antibodies to two different phosphorylation sites in ErbB2, Tyr877 and Tyr1248 (Figure 3a, b). Phosphorylation of Tyr877 is mediated by Src and contributes to the activation of the ErbB2 tyrosine kinase, and Tyr1248 is an autophosphorylation site [24,25]. Increased levels of ErbB3 (Figure 3e), Tyr1289-phosphorylated ErbB3 (Figure 3d), epidermal growth factor receptor (EGFR) (Figure 3f) and ErbB4 (Figure 3g) were readily detected in the c-ErbB2 tumours compared with normal tissue from the same mice.

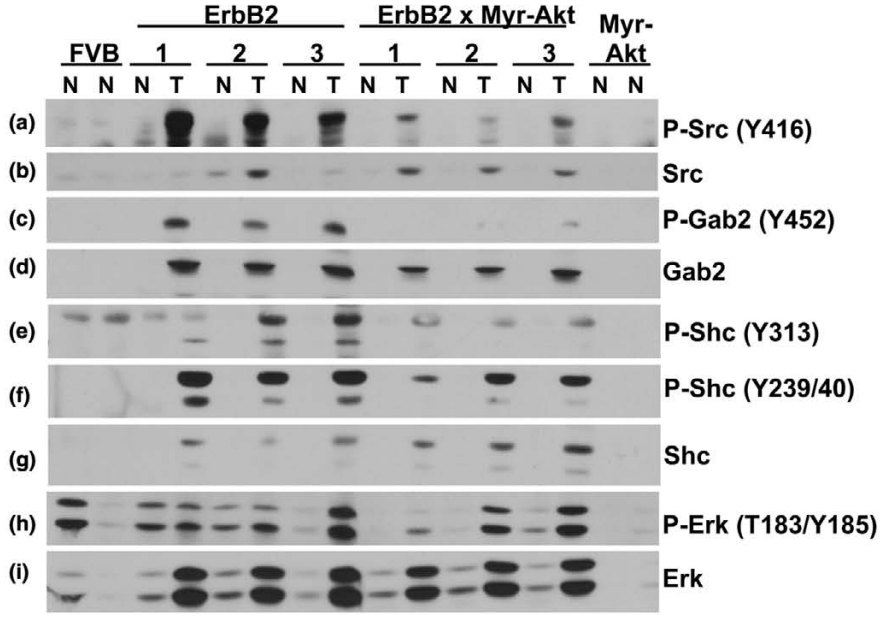
While increased levels of EGFR and ErbB3 protein could be detected in the bitransgenic tumours when compared with normal tissue, the extent of the increase was dramatically reduced compared with that observed in the c-ErbB2 tumours. Most importantly, the phosphorylation of ErbB3 was very low at Tyr1289 (Figure 3d), which is an important phosphorylation site for PI3K recruitment and activation [26]. ErbB4, the sole member of the EGFR family with an expression that bears positive prognostic value to breast cancer patients and suppresses proliferation and promotes apoptosis [27,28], was not detected in the bitransgenic tumours (Figure 3g). These data suggest that expression of activated Akt1 in the MMTV-c-ErbB2 transgenic mice alters the requirement for overexpression and activation of ErbB3 and other EGFR family members in mammary tumours induced by ErbB2.

Diminished activation of signalling downstream of ErbB2 in bitransgenic tumours

Mammary tumours expressing c-ErbB2 have been found to possess elevated levels of Src tyrosine protein kinase activity [29,30]. The Src protein binds to phosphotyrosine residues in the cytoplasmic tail of ErbB2, resulting in Src phosphorylation at Tyr416 and catalytic activation [31,32]. The amount of Tyr416-phosphorylated Src present in the c-ErbB2 tumours was dramatically increased in comparison to the bitransgenic tumours and normal tissue (Figure 4a), even though the bitransgenic tumours express more total Src than the c-ErbB2 tumours (Figure 4b). The reduced Src activation indicated by low Tyr416 phosphorylation levels in the bitransgenic tumours is corroborated by the reduced phosphorylation of ErbB2 at Tyr877 (Figure 3a), which is mediated by Src [24,25]. The reciprocal positive regulation of Src by ErbB2 and ErbB2 by Src is strongly diminished in the bitransgenic tumours (in addition to the reduced activation of ErbB3) indicating a general decline in plasma membrane tyrosine kinase signalling in the bitransgenic tumours.

The lack of activation/overexpression of EGFR family members and the lack of activation of Src in the bitransgenic tumours suggested that signalling mediated by secondary signalling molecules might also be attenuated in the bitransgenic tumours when compared with c-ErbB2 tumours. Gab2 is a scaffolding protein that can recruit the p85 subunit of PI3K when it is phosphorylated at Tyr452 [33] which in turn activates PH-domain containing proteins such as Akt [34]. The levels of Gab2 protein are elevated in both c-ErbB2 tumours

Figure 4



Tumours from MMTV-myr-Akt1, MMTV-c-ErbB2 mice have decrease tyrosine kinase signalling compared with tumours from MMTV-c-ErbB2 mice. Lysates from tumour (T) and normal mammary tissue (N) were used for immunoblot analysis using the following antibodies: **(a)** anti-phospho-Src (Tyr416); **(b)** anti-Src; **(c)** anti-phospho-Gab2 (Tyr452); **(d)** anti-Gab2; **(e)** anti-phospho-Shc (Tyr313); **(f)** anti-phospho-Shc (Tyr239/240): **(g)** anti-Shc; **(h)** anti-phospho-Erk (corresponding to Erk2 phosphorylation at Thr183 and Tyr185); and **(i)** anti-Erk.

and bitransgenic tumours compared with normal gland controls (Figure 4d), although the level is slightly higher in c-ErbB2 tumours than bitransgenic tumours. The amount of Tyr452-phosphorylated Gab2 is dramatically higher in the c-ErbB2 tumours compared with the bitransgenic tumours (Figure 4c) suggesting that expression of activated Akt1 in the bitransgenic animals attenuates phosphorylation of Gab2 and subsequent docking of PI3K, which occurs in c-ErbB2 animals.

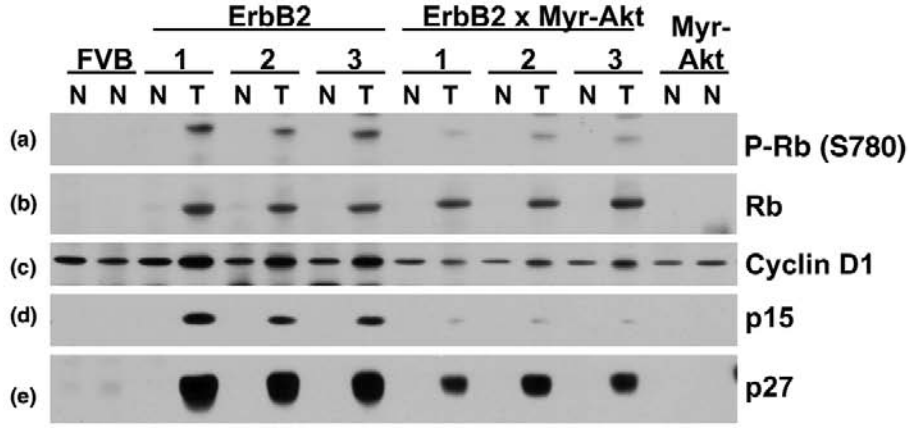
It has recently been demonstrated that another secondary signalling molecule, ShcA, is required for ErbB2-mediated tumourigenesis and phosphorylation of Tyr313 in ShcA may be important for tumour cell survival, while phosphorylation of Tyr239/240 may be important for tumour vascularisation [35]. c-ErbB2 tumours and bitransgenic tumours both demonstrate expression of ShcA (Figure 4g), particularly the p52 and the p46 isoforms while the p66 isoform was barely detectable. Both tumour types also appear to have similar levels of Tyr239/240-phosphorylated ShcA (with the bitransgenic tumours having slightly less phosphorylation than the c-ErbB2 tumours), indicating that activities regulated by phosphorylation of this site (perhaps in angiogenesis) are important in both tumour types (Figure 4f). However, the difference in phosphorylation of ShcA at Tyr313 is more dramatic between the two tumour types (Figure 4e), with the c-ErbB2 tumours having more ShcA phosphorylated at Tyr313 than the bitransgenic tumours suggesting that activities regulated by phosphorylation of this site (cell survival) may be less important in the tumours that express activated Akt1.

Oncogenic signalling often activates the Erk pathway, which is a known mediator of cell proliferation, cell survival, angiogenesis and cell migration (reviewed in [36]). Dual phosphorylation of Erk1 and Erk2 (corresponding to Thr183 and Tyr185 of human Erk2) in the activation loop results in catalytic activation of kinase activity [37]. Despite the attenuation of several signalling events in the bitransgenic tumours when compared with the c-ErbB2 tumours as discussed above, both tumour types maintain similar levels of phosphorylated/activated Erk (Figure 4h) indicating that transgenic activation of Akt1 does not bypass Erk signalling. Normal gland controls demonstrate various levels of Erk activation.

Expression/activation of cell cycle control proteins in bitransgenic tumours

The decrease in the tumour latency observed in the bitransgenic tumours would predict that there would be a change in the expression/modification of cell cycle control proteins in the bitransgenic tumours compared with the c-ErbB2 tumours. Phosphorylation of Rb by active cyclin-dependent kinases inactivates Rb activity and is an important regulatory step in cell cycle entry [38,39]. Immunoblot analysis with a phospho-specific antibody against Rb (Ser780) demonstrated Rb phosphorylation in tumour lysates from both genotypes of mice and not in the normal tissue from the same mice (Figure 5a). The extent of Rb phosphorylation was greater in the c-

Figure 5



MMTV-c-ErbB2 tumours demonstrate different cell cycle proteins than tumours from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice. Lysates from tumour (T) and normal mammary tissue (N) were used for immunoblot analysis using the following antibodies: **(a)** anti-phospho-Rb (Ser780); **(b)** anti-Rb; **(c)** anti-cyclin D1; **(d)** anti-p15; and **(e)** anti-p27.

ErbB2 tumours than in the bitransgenic tumours. This is surprising because the bitransgenic tumours have a shorter tumour latency and greater mitotic index which we predicted would correlate with more Rb phosphorylation and a more active cell cycle. However, Hutchinson *et al.* observed a similar phenomenon in their study: the NDL2 tumours (activated ErbB2) demonstrated a higher level of Ser780-phosphorylated Rb than the bitransgenic NDL2/Akt1DD tumours even though the bitransgenic animals had a shorter tumour latency [15]. The total amount of Rb is almost the same in all tumours examined indicating differences observed in Rb phosphorylation are not in fact due to differences in total Rb levels (Figure 5b).

We also examined a second cell cycle control protein, cyclin D1. The D-type cyclins are synthesised during the G1 phase of the cell cycle after stimulation of cells with growth factors that stimulate cell cycle entry and are required for cell cycle progression [40]. Immunoblot analysis revealed cyclin D1 in all samples with the bitransgenic tumours having a higher level of cyclin D1 than normal tissue, but the c-ErbB2 tumours containing more cyclin D1 than the bitransgenic tumours (Figure 5c). This data correlates with the Rb phosphorylation data which is consistent with the role of cyclin D1 in positively regulating cyclin dependent kinase activity and phosphorylation of Rb [40].

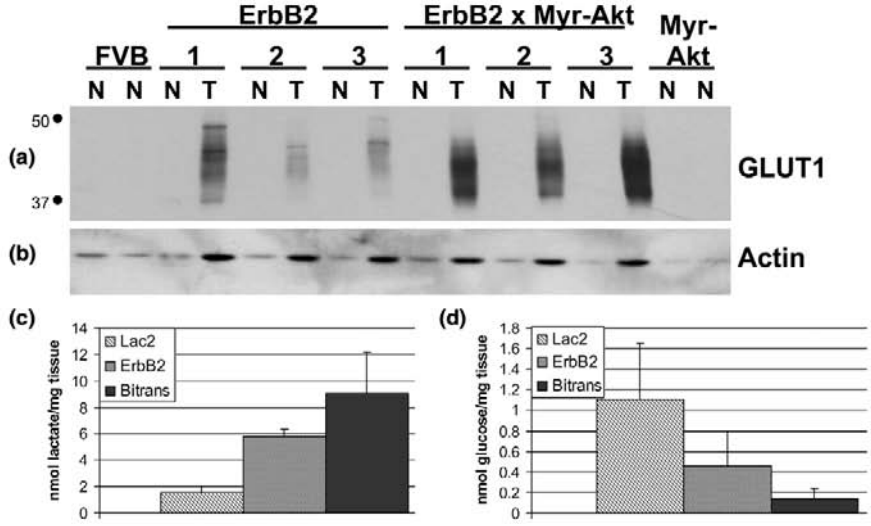
The bitransgenic tumours have a shorter latency than the c-ErbB2 tumours, but contain less phosphorylated Rb and less cyclin D1, which led us to examine some cell cycle inhibitors. p15 (INK4b), p27 (Kip1) and p21 (Cip1) are all inhibitors of cell cycle progression [41]. Immunoblot analysis for p15 demonstrated high expression in the c-ErbB2 tumours, a lack of expression in all normal glands and very faint expression in the bitransgenic tumours (Figure 5d). The expression profile of p27 was similar with a lack of expression in all normal tissue, high expression in c-ErbB2 tumours and slightly lower level of expression in the bitransgenic tumours (Figure 5e). We were unable to detect p53 and p21 in any of the tumour and normal mammary tissue samples, though both were present in an irradiated control gland (data not shown). While c-ErbB2 tumours contain more positive markers of the cell cycle than the bitransgenic tumours (phosphorylated Rb and cyclin D1), they also express more negative regulators of the cell cycle (p15 and p27), which likely serves to balance cell cycle progression.

An elevated metabolic phenotype in bitransgenic tumours compared with MMTV-c-ErbB2 tumours

One of the hallmarks of tumours and tumour cell lines is the elevated level of glucose transport to support the high glycolytic rate of tumour cells [42,43]. Myr-Akt1 can stimulate the translocation of the GLUT1 glucose transporter to the cell surface of lymphoid cells cultured in the absence of growth factors as well as increase hexokinase activity, glucose consumption and lactate production, which suggests that Akt stimulates aerobic glycolysis and produces the so-called 'Warburg effect' [44,45]. Immunoblot analysis demonstrates elevated levels of GLUT1 (the major glucose transporter in cancer cells) in lysates from mammary tumours from both the MMTV-c-ErbB2 and the bitransgenic mice compared with levels in normal glands. However, GLUT1 protein expression was greater in the bitransgenic tumours (Figure 6a). Cell-line transfection using GLUT1 cDNA validates the elongated band identified by GLUT1 immunoblot and the reason for this elongated band is probably due to glycosylation of GLUT1 (data not shown).

To determine whether the increased level of GLUT1 correlated with an increase in glycolysis (increased glucose consumption and increased lactate production), we used MRS to quantitate lactate concentrations in extracts prepared from tumours

Figure 6



Metabolic activity is elevated in tumours from bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice compared with tumours from MMTV-c-ErbB2 mice. Increased expression of the GLUT1 glucose transporter in bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 tumours compared with the MMTV-c-ErbB2 tumours. Lysates from tumour (T) and normal mammary tissue (N) were used for immunoblot analysis using a polyclonal antibody against the c-terminus of GLUT1 **(a)** and actin-loading control **(b)**. Intratumour concentration (nmol/mg tissue) of lactate **(c)** and glucose **(d)** in mammary glands at day 2 of lactation (L2), tumours from MMTV-c-ErbB2 and bitransgenic MMTV-myr-Akt1, MMTV-c-ErbB2 mice (all n = 5) calculated from ^{1}H-magnetic resonance spectroscopy.

because lactate is the end product of glycolysis. The mammary tumours examined in this study are of epithelial cell origin, so the use of mammary tissue from day two of lactation (L2) as a control tissue allows for the comparison of normal epithelium with tumour epithelium, whereas the normal virgin mammary gland is composed mostly of adipocytes [46]. Compared with normal L2 tissue, there was a four-fold increase in the amount of lactate in the MMTV-c-ErbB2 tumours and a six-fold increase in the bitransgenic tumours (Figure 6c) ($p < 0.00001$ between L2 and both tumours and $p < 0.02$ between ErbB2 and bitransgenic tumours, all $n = 5$). This increase in tumour glycolytic activity was accompanied by significantly decreased intratumuor concentrations of glucose: 1.10 nmol/mg in the L2 mammary gland, 0.46 nmol/mg in ErbB2 tumours ($p < 0.01$) and 0.14 nmol/mg in bitransgenic tumours ($p < 0.0004$) (Figure 6d). This indicates high glucose utilisation due to the increased activity of glycolytic enzymes. Thus, the bitransgenic tumours appear to consume more glucose and secrete more lactate than the c-ErbB2 tumours or the normal epithelial cell-enriched lactating mammary gland.

Discussion

We have demonstrated that expression of activated myr-Akt1 in MMTV-c-ErbB2 mice accelerates mammary tumourigenesis. Hutchinson *et al.* used a similar mammary tumourigenesis model using MMTV-NDL2-5 animals (which express activated ErbB2 in the mammary gland) and compared them with bitransgenic MMTV-NDL2-5, MMTV-Akt1DD animals (which express activated ErbB2 and an activated Akt1 gene that contains phosphomimetic mutations of Ser473 and Thr308) [15]. They found that expression of activated Akt1 accelerated ErbB2-induced tumourigenesis in their NDL2 model. The NDL2/Akt1DD bitransgenic tumours were more differentiated glandular tumours which expressed milk proteins. Expression of activated Akt1 in the MMTV-c-ErbB2 model did not induce more differentiated glandular tumours, but rather accelerated tumour formation often appearing to cause necrosis. Compared with the c-ErbB2 tumours, the bitransgenic tumours demonstrated half the amount of apoptosis and twice the amount of mitosis, suggesting that a decrease of apoptosis and increase in proliferation in the bitransgenic tumours contributed to faster tumour formation. Similar to Hutchinson *et al*., evaluation of apoptosis by TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining revealed almost no apoptotic cells in any of our tissue samples (data not shown), but evaluation of apoptosis by activated caspase-3 immunohistochemistry identified some apoptotic cells.

ErbB3, the major EGFR family member that activates the PI3K/Akt pathway [47], is required for ErbB2-induced cancer cell proliferation, transformation and colony formation *in vitro*[8,48]. There is a functional interaction between ErbB2 and ErbB3 in the MMTV-c-ErbB2 mouse model [8]. Activated Src is important for efficient ErbB2/ErbB3 heterocomplex formation and full activation of the ErbB2 kinase domain [24]. We have demonstrated that tumourigenesis in the MMTV-c-ErbB2 model proceeds with reduced activation of ErbB2, ErbB3, Src, Gab2 and Shc when activated Akt1 is expressed by transgene. Akt is often activated in cancer cells by the activating mutation of PI3K [49], inactivation of PTEN [50] and, a

recently demonstrated mechanism, by mutation of Akt itself [51]. Our results demonstrate that Akt activation by any of these means may lead to less dependency on ErbB2/ErbB3 and Src signalling without inhibiting tumourigenesis. It is not clear whether the diminished activation of these signalling molecules reflects a reduced need for them to be activated due to the presence of activated Akt or a negative feedback loop by which activated Akt can suppress activation of ErbB3-dependent signalling.

Two recent reports studying tumourigenesis in MMTV-ErbB2 mice on an Akt1-/- background highlight the importance of Akt1 in ErbB2-induced tumourigenesis [52,53]. Both groups used activated ErbB2 models of tumourigenesis in Akt1-/- and Akt+/+ backgrounds and demonstrated the importance of Akt1 in mediating ErbB2-induced tumourigenesis: mice lacking Akt1 either failed to develop tumours or tumourigenesis was delayed. However, while Akt1 is required for efficient tumour formation in MMTV-ErbB2 mouse models, activation of Akt1 alone is not sufficient for mammary tumourigenesis [11,12] indicating that other pathways downstream of ErbB2 activation are important for tumour formation.

The Erk pathway is activated in many types of cancer and can be activated by numerous oncogenic signals, including ErbB2 (Figure 4h) [36]. The bitransgenic tumours used in the current study maintained Erk activation (despite the loss of numerous other signalling events), which suggests that Erk signalling is necessary in these tumours. This may be one explanation for why Akt is necessary for ErbB2 tumourigenesis [52,53], but is not sufficient for mammary tumourigenesis [11,12]: activation of Akt alone may fail to activate Erk.

Most tumour cells rely on increased glycolysis, even in the presence of available oxygen (the Warburg effect). It has been shown that p53, HIF-1 (hypoxia inducible factor 1), c-Myc as well as Akt can all upregulate glycolytic enzymes (often through inhibition of the mitochondrial tricarboxylic acid cycle) to trigger increased tumour cell glucose consumption [54]. The increased levels of lactate and reduced concentrations of glucose in both types of tumours in the present study is consistent with an increased glycolytic rate in the tumours, but the bitransgenic tumours contained more lactate and more GLUT1 than the c-ErbB2 tumours. This led to the hypothesis that activation of Akt1 induced aerobic glycolysis in these mammary carcinomas. This may offer another explanation for the accelerated tumour onset in the bitransgenic animals because glycolysis, while inefficient in terms of wasting the full oxidative potential of glucose, is efficient at producing ATP and also generating metabolic byproducts necessary for membrane synthesis [55].

Human breast carcinomas overexpress ErbB2 in 20% to 30% of cases and patients with this type of tumour bear a poor prognosis and are currently treated with trastuzumab, a humanised monoclonal antibody. Trastuzumab treatment significantly boosts the prognosis for patients with breast tumours that overexpress ErbB2, but the response rate for these patients is about 50% to 60% [56]. Nagata *et al.* demonstrated that loss of the PTEN tumour suppressor (which induces Akt activation) predicts resistance to trastuzumab treatment [57]. We predict that our bitransgenic animals represent a model of a patient who would be resistant to trastuzumab. Tumours from bitransgenic animals overexpress ErbB2, making them candidates for ErbB2-targeted therapy, but drugs which molecularly target ErbB2 (such as trastuzumab) or ErbB2/ErbB3 heterodimers (such as pertuzumab) may be futile because signalling downstream of ErbB2/ErbB3 is already attenuated in bitransgenic animals. If activation of Akt1 in human tumours (which can occur by loss of PTEN function or activating mutation of either PI3K or Akt) is critical to ErbB2-positive breast cancer cells becoming trastuzumab resistant, then evaluation of the activation status of Akt may assist in deciding the prognosis and treatment strategy for breast cancer patients as compared with the current screening that only identifies amplification of ErbB2. Additionally, therapies which molecularly target the Akt pathway may be critical to overcome trastuzumab resistance.

Conclusion

Expression of activated Akt1 in the mammary gland of MMTV-c-ErbB2 mice accelerates tumourigenesis and attenuates signalling events sometimes thought to be critical to tumourigenesis. The bitransgenic tumours also have an accelerated glucose metabolism. Our studies suggest that tumours that overexpress ErbB2 which activate Akt by means of mutating PI3K, PTEN or Akt (rather than via ErbB2/ErbB3 activation of PI3K) may be resistant to ErbB2-targeted therapies. Therefore, therapies which molecularly target signalling events downstream of ErbB2, such as those mediated by Akt, may prove to be valuable.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CDY performed the immunoblot analyses, interpreted data, assembled the figures and drafted the manuscript. EN quantitated apoptosis, performed *ERBB2* mutation analysis and assisted with preliminary studies. AL maintained the animal colony including breeding, genotyping, tumour palpation and tissue harvest. NJS performed MRS analysis and interpreted metabolic data. SMA conceived the study and directed the research. NJS and SMA critically reviewed the manuscript, which was approved by all authors.

Acknowledgements

The authors would like to thank Dr Jeff Holt, Dr Ann Thor and Susan Edgerton for consultation on mammary gland and tumour histology. The authors also thank the members of the Mammary Gland Program Project Grant at the University of Colorado Denver for discussions and encour-

agement during this research. This work was supported by the grants BCTR0600727 from the Susan G. Komen Foundation, P01HD38129 from the Public Health Service to SMA and P30CA046934 Cancer Center Core grant to NJS. CDY is supported by a Predoctoral Fellowship from the US Army Breast Cancer Research Program (BC051149).

References

1. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL: **Human breast cancer: correlation of relapse and survival with amplification of the *HER/neu* oncogene.** *Science* 1987, **235:**177-182.
2. Slamon DJ, Godolphin W, Jones LA, Holt JA, Wong SG, Keith DE, Levin WJ, Stuart SG, Udove J, Ullrich A, Press MF: **Studies of the *HER-2/neu* proto-oncogene in human breast and ovarian cancer.** *Science* 1989, **244:**707-712.
3. Muller WJ, Sinn E, Pattengale PK, Wallace R, Leder P: **Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-neu oncogene.** *Cell* 1988, **54:**105-115.
4. Guy CT, Cardiff RD, Muller WJ: **Activated neu induces rapid tumor progression.** *Journal of Biological Chemistry* 1996, **271:**7673-7678.
5. Bouchard L, Lamarre L, Tremblay PJ, Jolicoeur P: **Stochastic appearance of mammary tumors in transgenic mice carrying the MMTV/c-neu oncogene.** *Cell* 1989, **57:**931-936.
6. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ: **Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease.** *Proc Natl Acad Sci USA* 1992, **89:**10578-10582.
7. Siegel PM, Ryan ED, Cardiff RD, Muller WJ: **Elevated expression of activated forms of Neu/ErbB-2 and ErbB-3 are involved in the induction of mammary tumors in transgenic mice: implications for human breast cancer.** *EMBO Journal* 1999, **18:**2149-2164.
8. Kim A, Liu B, Ordonez-Ercan D, Alvarez KM, Jones LD, McKimmey C, Edgerton SM, Yang X, Thor AD: **Functional interaction between mouse erbB3 and wild-type rat c-neu in transgenic mouse mammary tumor cells.** *Breast Cancer Res* 2005, **7:**R708-R718.
9. Holbro T, Beerli RR, Maurer F, Koziczak M, Barbas CF III, Hynes NE: **The ErbB2/ErbB3 heterodimer functions as an oncogenic unit: ErbB2 requires ErbB3 to drive breast tumor cell proliferation.** *Proc Natl Acad Sci USA* 2003, **100:**8933-8938.
10. Guy PM, Platko JV, Cantley LC, Cerione RA, Carraway KL III: **Insect cell-expressed p180erbB3 possesses an impaired tyrosine kinase activity.** *Proc Natl Acad Sci USA* 1994, **91:**8132-8136.
11. Schwertfeger KL, Richert MM, Anderson SM: **Mammary gland involution is delayed by activated Akt in transgenic mice.** *Mol Endocrinol* 2001, **15:**867-881.
12. Hutchinson J, Jin J, Cardiff RD, Woodgett JR, Muller WJ: **Activation of Akt (protein kinase B) in mammary epithelium provides a critical cell survival signal required for tumor progression.** *Mol Cell Biol* 2001, **21:**2203-2212.
13. Ackler S, Ahmad S, Tobias C, Johnson MD, Glazer RI: **Delayed mammary gland involution in MMTV-AKT1 transgenic mice.** *Oncogene* 2002, **21:**198-206.
14. Bellacosa A, Tests JR, Staal SP, Tsichlis PN: **A retroviral oncogene, *akt*, encoding a serine threonine kinase containing an SH2-like region.** *Science* 1991, **254:**274-277.
15. Hutchinson JN, Jin J, Cardiff RD, Woodgett JR, Muller WJ: **Activation of Akt-1 (PKB-alpha) can accelerate ErbB-2-mediated mammary tumorigenesis but suppresses tumor invasion.** *Can Res* 2004, **64:**3171-3178.
16. Dourdin N, Schade B, Lesurf R, Hallett M, Munn RJ, Cardiff RD, Muller WJ: **Phosphatase and tensin homologue deleted on chromosome 10 deficiency accelerates tumor induction in a mouse model of ErbB-2 mammary tumorigenesis.** *Can Res* 2008, **68:**2122-2131.
17. Muller WJ, Arteaga CL, Muthuswamy SK, Siegel PM, Webster MA, Cardiff RD, Meise KS, Li F, Halter SA, Coffey RJ: **Synergistic interaction of the neu proto-oncogene product and transforming growth factor a in the mammary epithelium of transgenic mice.** *Mol Cell Biol* 1996, **16:**5726-5736.
18. Siegel PM, Dankort WL, Hardy WR, Muller WJ: **Novel activating mutations in the *neu* proto-oncogene involved in induction of mammary tumors.** *Mol Cell Biol* 1994, **14:**7068-7077.
19. Rudolph MC, McManaman JL, Phang T, Russel T, Kominsky DM, Serkova N, Anderson SM, Neville MC: **Metabolic regulation in the lactating mammary gland: a lipid synthesizing machine.** *Physiol Genomics* 2007, **28:**323-336.
20. Andrechek ER, Muller WJ: **Tyrosine kinase signalling in breast cancer. Tyrosine kinase-mediated signal transduction in transgenic mouse models of human breast cancer.** *Breast Cancer Res* 2000, **2:**211-216.
21. Kim H, Muller WJ: **The role of the epidermal growth factor receptor family in mammary tumorigenesis and metastasis.** *Exp Cell Res* 1999, **253:**78-87.
22. Siegel PM, Hardy WR, Muller WJ: **Mammary gland neoplasia: insights from transgenic mouse models.** *BioEssays* 2000, **22:**554-563.
23. Rosner A, Miyoshi K, Landesman-Bollag E, Xu X, Seldin DC, Moser AR, Macleod CL, Shyamala G, Gillgrass AE, Cardiff RD: **Pathway pathology: histological differences between ErbB/Ras and Wnt pathway transgenic mammary tumors.** *Am J Pathol* 2002, **161:**1087-1097.
24. Ishizawar RC, Miyake T, Parsons SJ: **c-Src modulates ErbB2 and ErbB3 heterocomplex formation and function.** *Oncogene* 2007, **26:**3503-3510.
25. Xu W, Yuan X, Beebe K, Xiang Z, Neckers L: **Loss of Hsp90 association up-regulates Src-dependent ErbB2 activity.** *Mol Cell Biol* 2007, **27:**220-228.
26. Schulze WX, Deng L, Mann M: **Phosphotyrosine interactome of the ErbB-receptor kinase family.** *Mol Syst Biol* 2005, **1:**2005.
27. Sartor CI, Zhou H, Kozlowska E, Guttridge K, Kawata E, Caskey L, Harrelson J, Hynes N, Ethier S, Calvo B, Earp HS III: **Her4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells.** *Mol Cell Biol* 2001, **21:**4265-4275.
28. Naresh A, Long W, Vidal GA, Wimley WC, Marrero L, Sartor CI, Tovey S, Cooke TG, Bartlett JM, Jones FE: **The ERBB4/HER4 intracellular domain 4ICD is a BH3-only protein promoting apoptosis of breast cancer cells.** *Cancer Res* 2006, **66:**6412-6420.
29. Olayioye MA, Badache A, Daly JM, Hynes NE: **An essential role for Src kinase in ErbB receptor signaling through the MAPK pathway.** *Exp Cell Res* 2001, **267:**81-87.
30. Muthuswamy SK, Siegel PM, Dankort DL, Webster MA, Muller WJ: **Mammary tumors expressing the neu proto-oncogene possess elevated c-src tyrosine kinase activity.** *Mol Cell Biol* 1994, **14:**735-743.
31. Xu W, Doshi A, Lei M, Eck MJ, Harrison SC: **Crystal structures of c-src reveal features of its autoinhibitory mechanism.** *Molecular Cell* 1999, **3:**629-638.
32. Mayer BJ: **Signal transduction: clamping down on Src activity.** *Current Biology* 1997, **7:**R295-R298.
33. Crouin C, Arnaud M, Gesbert F, Camonis J, Bertoglio J: **A yeast two-hybrid study of human p97/Gab2 interactions with its SH2 domain-containing binding partners.** *FEBS Lett* 2001, **495:**148-153.
34. Manning BD, Cantley LC: **AKT/PKB signaling: navigating downstream.** *Cell* 2007, **129:**1261-1274.
35. Ursini-Siegel J, Hardy WR, Zou D, Lam SHL, Sanguin-Gendreau V, Cardiff RD, Pawson T, Muller WJ: **ShcA signaling is essential for tumour progression in mouse models of human breast cancer.** *EMBO Journal* 2008, **27:**910-920.
36. Roberts PJ, Der CJ: **Targeting the Raf-MEK-ERK mitogen-activated protein kinase cascade for the treatment of cancer.** *Oncogene* 2007, **26:**3291-3310.
37. Anderson NG, Maller JL, Tonks NK, Sturgill TW: **Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase.** *Nature* 1990, **343:**651-653.
38. Decaprio JA, Furukawa Y, Ajchenbaum F, Griffin JD, Livingston DM: **The retinoblastoma-susceptibility gene product becomes phosphorylated in multiple stages during cell cycle entry and progression.** *Proc Natl Acad Sci USA* 1992, **89:**1795-1798.
39. Goodrich DW, Wang NP, Qian Y-W, Lee EYHP, Lee W-H: **The retinoblastoma gene product regulates progression through the G1 phase of the cell cycle.** *Cell* 1991, **67:**293-302.
40. Sherr CJ, Roberts JM: **Living with or without cyclins and cyclin-dependent kinases.** *Genes and Dev* 2004, **18:**2699-2711.

41. Caldon CE, Daly RJ, Sutherland RL, Musgrove EA: **Cell cycle control in breast cancer cells.** *J Cell Biochem* 2006, **97:**261-274.
42. Hatanaka M: **Transport of sugars in tumor cell membranes.** *Biochim Biophys Acta* 1974, **355:**77-104.
43. Warburg O: **On the origin of cancer cells.** *Science* 1956, **123:**309-314.
44. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB: **Akt stimulates aerobic glycolysis in cancer cells.** *Cancer Res* 2004, **64:**3892-3899.
45. Rathmell JC, Fox CJ, Plas DR, Hammerman PS, Cinalli RM, Thompson CB: **Akt-directed glucose metabolism can prevent Bax conformation change and promote growth factor-independent survival.** *Mol Cell Biol* 2003, **23:**7315-7328.
46. Richert MM, Schwertfeger KL, Ryder JW, Anderson SM: **An atlas of mouse mammary gland development.** *J Mam Gland Bio Neo* 2000, **5:**227-241.
47. Olayioye MA, Neve RM, Lane HA, Hynes NE: **The ErbB signaling network: receptor heterodimerization in development and cancer.** *EMBO Journal* 2000, **19:**3159-3167.
48. Liu B, Ordonez-Ercan D, Fan Z, Edgerton SM, Yang X, Thor AD: **Downregulation of erbB3 abrogates erbB2-mediated tamoxifen resistance in breast cancer cells.** *Int J Cancer* 2007, **120:**1874-1882.
49. Bader AG, Kang S, Vogt PK: **Cancer-specific mutations in PIK3CA are oncogenic *in vivo*.** *Proceedings of the National Academy of Sciences* 2006, **103:**1475-1479.
50. Parsons R, Simpson L: **PTEN and cancer.** *Methods Mol Biol* 2003, **222:**147-166.
51. Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, Hostetter G, Boguslawski S, Moses TY, Savage S, Uhlik M, Lin A, Du J, Qian YW, Zeckner DJ, Tucker-Kellogg G, Touchman J, Patel K, Mousses S, Bittner M, Schevitz R, Lai MH, Blanchard KL, Thomas JE: **A transforming mutation in the pleckstrin homology domain of AKT1 in cancer.** *Nature* 2007, **448:**439-444.
52. Maroulakou IG, Oemler W, Naber SP, Tsichlis PN: **Akt1 ablation inhibits, whereas Akt2 ablation accelerates, the development of mammary adenocarcinomas in mouse mammary tumor virus (MMTV)-ErbB2/neu and MMTV-polyoma middle T transgenic mice.** *Cancer Res* 2007, **67:**167-177.
53. Ju X, Katiyar S, Wang C, Liu M, Jiao X, Li S, Zhou J, Turner J, Lisanti MP, Russell RG, Mueller SC, Ojeifo J, Chen WS, Hay N, Pestell RG: **Akt1 governs breast cancer progression *in vivo*.** *Proc Natl Acad Sci USA* 2007, **104:**7438-7443.
54. Young CD, Anderson SM: **Sugar and fat – that's where it's at: metabolic changes in tumors.** *Breast Cancer Res* 2008, **10:**202.
55. Hatzivassiliou G, Zhao F, Bauer DE, Andreadis C, Shaw AN, Dhanak D, Hingorani SR, Tuveson DA, Thompson CB: **ATP citrate lyase inhibition can suppress tumor cell growth.** *Cancer Cell* 2005, **8:**311-321.
56. Hudis CA: **Trastuzumab – mechanism of action and use in clinical practice.** *N Engl J Med* 2007, **357:**39-51.
57. Nagata Y, Lan KH, Zhou X, Tan M, Esteva FJ, Sahin AA, Klos KS, Li P, Monia BP, Nguyen NT: **PTEN activation contributes to tumor inhibition by trastuzumab, and loss of PTEN predicts trastuzumab resistance in patients.** *Cancer Cell* 2004, **6:**117-127.